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December 30, 2005

CERTIFICATE OF MAILING
37 C.F.R. 1.8

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December 30, 2005 — MDeLaPaz
Date — Monica A. De La Paz

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re: *SN 09/989,695 "LIPOSOMAL IMEXON" by Gabriel Lopez-Berestein et al.*
Our Ref. UTSC:648US; Client Ref. MDA00-024

Commissioner:

Enclosed for filing in the above-referenced patent application please find:

1. A Supplemental Brief on Appeal (including Appendices 1–3 and Exhibits 1–7);

2. A return postcard to acknowledge receipt of these materials. Please date stamp and mail this postcard.

No fees are believed due. However, should any additional fees be required under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/UTSC:648US.

Respectfully submitted,

MDeLaPaz

Monica A. De La Paz, MD ~~JD~~
Reg. No. 54,662

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Enclosures

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AF/1615 ZZW

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Gabriel LOPEZ-BERESTEIN, William A. REMERS and Evan HERSH.

Serial No.: 09/989,695

Filed: November 20, 2001

For: LIPOSOMAL IMEXON

Group Art Unit: 1615

Examiner: G. Kishore

Atty. Dkt. No.: UTSC:648US

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December 30, 2005
Date

Monica A. De La Paz

SUPPLEMENTAL BRIEF ON APPEAL

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: 1615
Gabriel LOPEZ-BERESTEIN, William A. REMERS and Evan HERSH.	Examiner: G. Kishore
Serial No.: 09/989,695	Atty. Dkt. No.: UTSC:648US
Filed: November 20,2001	
For: LIPOSOMAL IMEXON	

SUPPLEMENTAL BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Supplemental Appeal Brief in response to the Final Office Action dated July 14, 2004 and the Order Returning Undocketed Appeal to Examiner dated November 30, 2005. The Notice of Appeal was mailed November 15, 2004.

It is believed that no fee for filing this Supplemental Appeal Brief is due. The fee for filing the Appeal Brief was submitted with the Appeal Brief mailed on January 18, 2005. No additional fees are believed due in connection with the instant paper. However, should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/UTSC:648US. Please date stamp and return the enclosed postcard to evidence receipt of this document.

I. REAL PARTIES IN INTEREST

The real party in interest is the assignee, The Board Of Regents, The University Of Texas System (assignee) and Interpath, Inc. (licensee).

II. RELATED APPEALS AND INTERFERENCES

There are currently no related appeals or interferences.

III. STATUS OF THE CLAIMS

The application was filed with original claims 1-32. No claims were added during prosecution. Claim 3 was canceled during prosecution. Thus, claims 1, 2, and 4-32 are pending. Each of the presently pending claims is subject to the present appeal. A copy of the appealed claims is attached as Appendix 1.

IV. STATUS OF AMENDMENTS

In the response to the Office Action dated November 14, 2004, Appellants submitted an Amendment canceling claim 3, and amending claims 1, 2, 4, 11, 14-24, and 30-32. The final Office Action dated July 14, 2004, acknowledged the amendment. In the response to the final Office Action dated July 14, 2004, claims 24 and 26 were amended. The Advisory Action dated November 11, 2004, indicated that the amendment submitted with the response to the final Office Action dated July 14, 2004, would be entered. No amendments were sought to the pending claims following the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed generally to novel pharmaceutical compositions that include an imexon or derivative of an imexon in combination with one or more phospholipids.

Specification, page 5, lines 2-5. These compositions are formulated to allow for an improved therapeutic index, biodistribution, and/or aqueous solubility of the imexon or imexon derivative. Specification, page 5, lines 1-8. In some embodiments, the phospholipids are comprised in liposomes. Specification, page 5, lines 30-31. The derivative of imexon, in certain embodiments, is AMP-404. Specification, page 6, lines 3-4. In some embodiments, the pharmaceutical composition further comprises a targeting agent, a diagnostic agent, or a second therapeutic agent. Specification, page 6, lines 15-16. The present invention is also generally directed to pharmaceutical compositions that include an imexon or imexon derivative in combination with one or more phospolipids (Specification, page 5, lines 9-10 and 20). The present invention is also generally directed to methods of treating an individual with cancer, involving administering to the individual a therapeutically effective amount of a composition that includes an imexon or derivative thereof in combination with one or more phospholipids (Specification, page 5, lines 13-16), and methods of stimulating the immune system of an individual that involve administering a therapeutically effective amount of a composition that includes an imexon or derivative thereof in combination with one or more phospholipids (Specification, page 5, lines 17-19).

VI. ISSUES TO BE REVIEWED ON APPEAL

This appeal presents the following issues:

a) Whether the subject matter of claim 2 is adequately described in the subject specification as required by the written description requirement of 35 U.S.C. §112, first paragraph;

b) Whether the subject matter of claims 1-2 and 4-32 is obvious under 35 U.S.C. §103(a) over the combination of Hermann (Exhibit 1), further in view of either Sugarman

et al. (Exhibit 2), Ranade (Exhibit 3), Mayer *et al.* (Exhibit 4), or Weiner *et al.* (Exhibit 5);

c) Whether the subject matter of claims 1-2 and 4-32 is obvious under 35 U.S.C. §103(a) over the combination of WO 99/00120 (Exhibit 6), further in view of Sugarman *et al.*, Ranade, Mayer *et al.*, or Weiner *et al.*;

d) Whether the subject matter of claims 1-2 and 4-32 is obvious under 35 U.S.C. §103(a) over the combination of Hermann or WO '120, in view of Presant (Exhibit 7).

VII. ARGUMENT

A. Rejection of Claims under 35 U.S.C. §112, 1st Paragraph

The Advisory Action dated November 1, 2004, did not specify whether the rejection of claim 2 under 35 U.S.C. §112, first paragraph, had been overcome. Appellants herein will respond to this rejection under the assumption that it has not been overcome.

In the final Office Action, claim 2 was rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement because there is said to be no support in the specification for "micelles comprising phospholipids."

1. The subject matter of claim 2 is adequately described in the specification with respect to the phrase "micelles comprising phospholipids."

The objective standard for determining compliance with the written description requirement is whether "the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *Manual of Patent Examining Procedure* (*MPEP*), §2163.02, citing *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989). To clearly satisfy the written description requirement, Appellants must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, they were in possession of the invention, and that the invention, in that context, is whatever is now claimed.

MPEP, §2163.02, citing *Vas-Cath, Inc., v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). Appellants shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structure, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

The specification provides substantial detail regarding embodiments of the present invention that pertain to "micelles comprising phospholipids." Specifically, the specification provides that "[i]n certain embodiments, the lipid or lipids comprise at least one phospholipid." Specification, page 5, lines 20-21. Particular examples of phospholipids are provided thereafter. See Specification, page 5, lines 20-29. In addition, a detailed discussion concerning phospholipids can be found in the specification on page 26, lines 8-26. In view of these sections of the specification, one of ordinary skill in the art would understand that phospholipids are encompassed by the term "lipids" in the context of the present invention.

Furthermore, the specification clearly conveys that in certain embodiments, at least a portion of the lipids could comprise micelles. In particular, the specification clearly states that **"[i]n certain embodiments, at least a portion of the lipids comprise micelles."** Specification, page 5, line 30 (emphasis added). The specification also provides that:

> An imexon and/or a derivative thereof associated with a lipid may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid or otherwise associated with a lipid. A lipid or lipid/imexon and/or a derivative thereof associated composition of the present invention is not limited to any particular structure. For example, they may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape. In another example, they may be present in a bilayer structure, as **micelles**, or with a "collapsed" structure.

Specification, page 28, lines 16-23 (emphasis added). In addition, the specification contains an entire section pertaining to micelles and lipid compositions that include micelles. In particular, the specification provides that:

> **A lipid may be comprised in a micelle.** A **micelle** is a cluster or aggregate of lipid compounds, generally in the form of a lipid monolayer, and may be prepared using any micelle producing protocol known to those of skill in the art (*e.g.*, Canfield *et al.*, 1990; El-Gorab *et al.*, 1973; Colloidal Surfactant, 1963; and Catalysis in Micellar and Macromolecular Systems, 1975, each incorporated herein by reference). For example, one or more lipids are typically made into a suspension in an organic solvent, the solvent is evaporated, the lipid is resuspended in an aqueous medium, sonicated and then centrifuged.

Specification, page 30, lines 8-15 (emphasis added). Thus, the specification provides that (1) phospholipids are a type of lipid that can be used in the context of the present invention, and (2) lipids, as the term "lipid" is defined herein, may be comprised in a micelle. Therefore, one of ordinary skill in the art, upon reading this specification, would understand that phospholipids may be comprised in a micelle. Therefore, the application clearly conveys compositions wherein at least a portion of the phospholipids are comprised in micelles. Consequently, one of ordinary skill in the art, upon reading the specification, would understand that the inventors had possession of the claimed invention.

Accordingly, there is ample written description support in the specification for present claim 2. Therefore, Appellants respectfully request that the Board withdrawn the written description rejections under 35 U.S.C. §112, first paragraph.

B. Rejection of Claims under 35 U.S.C. §103(a)

1. *Claims 1-2 and 4-32 are not Obvious over Hermann, further in view of either Sugarman et al., Ranade, Mayer et al., or Weiner et al.*

a) *The Examiner's Rejection*

The Examiner has rejected claims 1-2 and 4-32 under 35 U.S.C. §103(a) as being unpatentable over Hermann, further in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, or Weiner *et al.* The Examiner contends that Hermann discloses compositions containing imexon and a lipid, specifically magnesium stearate. Sugarman *et al.* is said to teach (1) that liposomes are sustained release agents and that they are advantageous as carriers of drugs because they reduce toxicity associated with those drugs; (2) the use of DMPC/DMPG in a ratio of 7:3; and (3) the attachment of monoclonal antibodies to the surface of liposomes to direct the liposomes to the target tissue as being known in the art. Ranade is said to disclose advantages of using liposomes as carriers of drugs, and the sustained release and site-specific release of drugs. Mayer *et al.* is said to teach the tumor uptake and anti-tumor efficacy of doxorubicin against murine mammary tumors. Weiner *et al.* is said to teach the advantages of using liposomes as carriers of drugs, and their sustained release and site-specific release of drugs.

According to the Examiner, the use of liposomes as carriers of imexon would have been obvious to one of ordinary skill in the art, and the motivation comes from the teachings of these references and from the knowledge available to one of ordinary skill in the art. Specifically, the Examiner contends that there is "clear motivation" in the secondary references for one of ordinary skill in the art to use liposomes for the delivery of imexon. See Advisory Action, page 2. Further, Sugarman *et al.* and Ranade are said to teach the advantages of using liposomes as sustained delivery agents for both hydrophobic and hydrophilic active agents, particularly cancer

agents, and Mayer *et al.* is said to show the increased uptake of the liposomes containing an anti-cancer drug by tumor cells. See Advisory Action, page 2.

Furthermore, the Examiner contends in the Advisory Action that liposomal art is well advanced in the sustained delivery of a variety of drugs and therefore, motivation to use liposomes comes from the knowledge available to one of ordinary skill in the art.

b) The Examiner's Burden to Establish a Prima Facie Case of Obviousness

In order to establish a *prima facie* case of obviousness, the Examiner must meet three basic criteria: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *Manual of Patent Examining Procedure (MPEP)* § 2142. See also *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991) (emphasizing that the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art, and not based on Appellant's disclosure). It is important to note that the Examiner must show all three elements to establish a *prima facie* case of obviousness. Thus, if one element is missing, a *prima facie* case of obviousness does not exist.

c) The Examiner has Failed to Establish a Prima Facie Case of Obviousness Because he has Failed to Identify Some Suggestion or Motivation to Combine Reference Teachings

In response, Appellants contend that the Examiner has failed to establish a *prima facie* case of obviousness because he has failed to identify some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art,

to combine the reference teachings. Because the Examiner has failed to establish this element, a *prima facie* case of obviousness has not been established.

The Examiner's assertion that the suggestion or motivation can be found in the cited prior art references is misplaced. Hermann does not disclose phospholipids or liposomes. Similarly, Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* make no reference to imexon.

"The mere fact that references can be combined or modified does not render the resultant combination obvious ***unless the prior art also suggests the desirability of the combination***." *MPEP* § 2143.01 (emphasis added). None of these references makes any suggestion of delivering imexon via administration of liposomes. Thus, the suggestion or motivation to combine reference teachings is not found in the cited prior art references.

Nor is there a suggestion or motivation to combine reference teachings from the knowledge generally available to one of ordinary skill in the art. To make such a showing, the Examiner must provide "objective evidence" and "specific factual findings with respect to the motivation to combine references." *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 13113, 1317 (Fed. Cir. 2000). No such showing has been made by the Examiner. The Examiner appears to be implying that the suggestion or motivation to combine reference teachings can be implicit in the knowledge generally available to one of ordinary skill, and that no factual findings or objective evidence are needed to make such a showing. However, this is an incorrect interpretation of case law that contravenes *In re Kotzab*. *In re Kotzab*, 217 F.3d. at 1370.

Appellants contend that many drug therapies have been investigated in treating cancer, but in two of the references that discuss liposomal drug delivery, Mayer *et al.* and Weiner *et al.*, only one drug is mentioned, doxorubicin. None of these references make any recommendations as to which cancer drugs may be suitable for liposomal delivery other than doxorubicin.

Sugarman *et al.* states that "most of the chemotherapeutic agents used have been doxorubicin or cisplatin derivatives." Sugarman *et al.* also mentions use of muramyl peptides with liposomes. Ranade discloses use of liposomes with doxorubicin, cisplatin, and macrophage activation factors. However, none of the drugs mentioned have any similarity or structural resemblance to imexon. Hundreds of drugs exist for treating cancer such that one skilled in the art could not possibly know that imexon would be a drug appropriate for liposomal delivery. Furthermore, since Hermann does not explicitly state that such use would be possible, one skilled in the art would be even less motivated to combine the references. Therefore, the suggestion or motivation to combine the teachings of these references to result in the claimed invention does not have its basis in the knowledge of one of ordinary skill in the art.

Appellants disagree with Examiner's contention that there is clear motivation to one of ordinary skill in the art to combine reference teachings. In support of this contention, the Examiner asserts that Sugarman *et al.*, and Ranade in particular teach the advantages of using liposomes as sustained delivery agents for both hydrophobic and hydrophilic active agents, cancer agents in particular and that of Mayer *et al.* shows the increased uptake of liposomes containing an anti-cancer drug by the tumor cells. However, as noted above, hundreds of drugs exist for treating cancer. One of ordinary skill in the art could not possibly know that imexon would be a drug appropriate for liposomal delivery, and the Examiner's unsupported conclusion to the contrary cannot be accepted merely at face value. Further, the Examiner's contention that "liposomal art is well advanced in the sustained delivery of a variety of drugs and therefore, motivation to use liposomes comes from the knowledge available to one of ordinary skill in the art" (Office Action, paragraph 1, page 5) fails to address the issue at hand, which is the question of delivery of a particular compound - imexon.

The Examiner has stated in the final Office Action that "the novelty is the sustained delivery nature of the liposomes themselves and this sustained delivery does not depend upon the drug encapsulated and therefore, one of ordinary skill in the art would expect at least the same results using imexon as the drug." Final Office Action, paragraph 1, page 5. The Examiner appears to be contending that use of imexon would be obvious since liposomes have been successfully used to deliver other types of drugs, and that this is sufficient to establish obviousness for the delivery of ***any*** drug. However, obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so, either explicity or implicity, found in the references themselves or in the art. *MPEP* §2143.01; *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000).

In *In re Kotzab*, the claims were drawn to an injection molding method using a single temperature sensor to control a plurality of flow control valves. The primary reference disclosed a multizone device having multiple sensors, each of which controlled an associated flow control valve, and also taught that one system may be used to control a number of valves. The court found that there was insufficient evidence to show that one system was the same as one sensor. While the control of multiple valves by a single sensor rather than by multiple sensors was a "technologically simple concept," there was no finding "as to the specific understanding or principle within the knowledge of the skilled artisan" that would have provided motivation to use a single sensor as to the system to control more than one valve. *In re Kotzab*, 217 F.3d at 1371.

In re Kotzab appears to apply in the facts at hand. The Examiner appears to be of the opinion that one of ordinary skill in the art would find that use of imexon in liposomes is obvious because of the success of liposomes in delivering other drugs, and is thus obvious. However, as

noted in *In re Kotzab*, such an assertion is not sufficient to render a case obvious. There must be some finding as to the specific understanding or principle within the knowledge of the skilled artisan that would have provided motivation to use imexon in liposomes. No such finding has been established, and thus the Examiner has failed to meet his burden of establishing a *prima facie* case of obviousness.

d) The Examiner has Failed to Establish a Prima Facie Case of Obviousness Because he has Failed to Identify a Reasonable Expectation of Success to Combine Teachings of the Cited References

Another element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of Hermann in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* would be successful. Appellants, in their previous response, have submitted that there would be no reasonable expectation of success in combining imexon and liposomal delivery in treating cancer. The Examiner has failed to provide any evidence that combining imexon and liposomes would reasonably result in success.

The result of combining drug therapies is impossible to predict. The Examiner appears to merely assume that combining imexon and liposomal drug delivery will automatically result in success. There simply is no basis for such an assumption. Furthermore, none of the five references provide any guidance or recommendations as to whether combining imexon with liposomal drug delivery would be successful in treating cancer. At best, the prior art presents an "obvious to try" situation. Specifically, the combination of imexon and liposomal delivery may or may not be successful. However, the PTO's reviewing court has consistently held that "'obvious to try' is not the standard" and "does not render a claim obvious." *Ecolochem, Inc. v. Southern California Edison Co.*, 227 F.3d 1361, 56 U.S.P.Q.2d 1065 (Fed. Cir. 2000), *In re Roemer*, 258 F.3d 1303, 59 U.S.P.Q.2d 1537 (Fed. Cir. 2001).

In view of the prior art, a person of ordinary skill in the art could not reasonably expect to achieve success in administering imexon via liposomal delivery. As a result, Hermann in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* does not establish a reasonable expectation of success as required for a *prima facie* case of obviousness.

Further, Presant, cited by the Examiner in the final Office Action as a case of interest, is said to teach that liposomes are also called micelles. This fails to address the missing element of expectation of success. Presant pertains to methods of targeting locations in a body using micellular particles. It contains no information pertaining to delivery of imexon via liposomal delivery.

e) The Pharmaceutical Compositions Set Forth in the Claims have Exceptionally Superior Activity Against Tumor Cells

Even if the Examiner had established a *prima facie* case of obviousness, which Appellants strongly assert that this has not been the case, the obviousness rejection could be readily overcome by Appellants' evidence, set forth in the Specification, of the unexpectedly superior activity of certain of the claimed pharmaceutical compositions against tumor cells. See *MPEP* §2144.09, citing *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963); *In re Weichert*, 370 F.2d 927, 152 USPQ 247 (CCPA 1967).

The anti-cancer activity of selected anti-cancer imexon analogs is addressed in Example 20 of the Specification. Specification, page 84, lines 1-24. In this example, three different analogs of imexon (AMP-404, AMP-415, and AMP-416) were tested in a cytotoxic assay against 822R myeloma cells. The drugs, AMP 404 DMPC-DMPG, AMP 404 DMPC-PA, AMP 404 DMPC, DMPC empty liposomes, AMP 404 alone, AMP 415 DMPC-DMPG, AMP 415 DMPC-PA, AMP 415 DMPC, and AMP 415 alone were tested for cytotoxicity against 8226R (myeloma cell line) using the MTT assay. The results were recorded as % cytotoxicity versus drug

concentration. FIG. 1A, FIG. 1B, and FIG. 1C, which pertain to AMP 404, demonstrates that the liposome formulations comprising AMP 404 were more potent that AMP 404 alone, particularly on day 1 (FIG. 1A) and day 2 (FIG. 1B). Similarly, for AMP 415, formulations of AMP 415 comprised in liposomes were more potent compared to AMP 415 alone, particularly at day 1 and day 2. See FIG. 2A (day 1), FIG. 2B (day 2), and FIG. 2C (day 3). Thus, liposome formulations of AMP 404 and AMP 415 have superior potency compared to either drug alone.

Furthermore, Example 21 (Specification, page 84, line 27 through page 85, line 16) summarizes the antitumor activity of a set of twenty 2-cyanoaziridine-1-carboxamides (summarized in Table 3, page 86 of the Specification). These compounds were tested against a panel of tumor cells in culture, and were active against a variety of solid and hematological tumor cells, including strains resistant to doxorubicin and mitoxantrone. Their potencies in these assays correlated with the lipophilicity of the substituents. In particular, the N-phenyl derivative was found to be more potent and equally effective compared to imexon against cloned fresh human tumors. The relative potencies of the N-substituted 2-cyanoaziridine-1-carboxamides in a panel of tumor cell cultures are compared in Table 3 (Specification, page 86).

As can be seen in Table 4, many of the 2-cyanoaziridine-1-carboxamides are more potent than imexon. For example, number 14, the 2-cyanoaziridine-1-(N-phenyl) carboxamide, is about 90 times more potent than imexon against ovarian carcinoma cells (OVCAR3), and about 30 times more potent than imexon against melanoma cells (A375). Addition of lipophilic groups to the amide nitrogen significantly increased the cytotoxicity and decreased the selectivity so that in many cases the compounds were roughly equipotent across the spectrum of tumor types. A statistically significant correlation was found (99% confidence limit) between antitumor potency for sensitive multiple myeloma and the lipophilicity of substituents as

represented by their contributions (π) to the octanol-water partition coefficients, using simple linear regressionand the program Sigmastat. Specification, page 87, lines 11-15. The data for this correlation is shown in Table 4. Specification, page 87-88. Furthermore, Table 5 (Specification, page 89) indicates that compound 13 of Table 4 at a concentration of 0.2 μM is about as effective as imexon at 1.0 μM. Although both imexon and compound 13 have similar profiles of activity at these concentration, compound 13 appears to be superior against the sarcoma cells. 2-cyanoaziridine-1-carboxamides with alkyl, aryl, and other substituents on the amide nitrogen have greater potency than the N-unsubstituted parent compound against tumor cells in culture. Specification, page 89, lines 6-8. This greater potency correlates with the lipophilicity of the substituents, which indicates that cell penetration may be an important factor in cytotoxicity. Specification, page 89, lines 8-10. Furthermore, N-phenyl derivative 13, was found to be more potent than imexon. Specification, page 89, lines 11-13. The results set forth in Example 20 pertaining to the increased potency of liposomal formulations of imexon derivatives compared to drug alone suggests that many of the 2-cyanoaziridine-1-carboxamides set forth in Example 21 may be even more potent when formulated with liposomes.

Therefore, Appellants contend that this evidence, which pertains to (1) the superior properties of liposome formulations of particular imexon analogs compared to imexon analog alone; and (2) evidence of the superior potency of imexon analogs against a panel of tumor cells in culture compared to imexon alone, is sufficient to rebut any contention that a *prima facie* case of obviousness has been established by the Examiner.

f) Conclusion

In view of the above arguments, Appellants have shown that the Examiner has not established a *prima facie* case that claims 1-2 and 4-32 were obvious at the time of filing. Furthermore, Appellants have set forth evidence that even if the Examiner had established a

prima facie case of obviousness, which Appellants assert has not been the case herein, that such a finding could be successfully overcome by the unexpectedly superior properties of the pharmaceutical compositions set forth above. Accordingly, Appellants respectfully request that the Board withdraw the rejection of claims 1, 2, and 4-32.

2. *Rejection of claims 1-2 and 4-32 as being unpatentable over WO 99/00120, further in view of Sugarman et al., Ranade, Mayer et al., or Weiner et al.*

a) *The Examiner's Rejection*

Claims 1, 3-32 stand rejected under 35 U.S.C. 103(a) in the final Office Action dated July 14, 2004, as being unpatentable over WO '120, further in view of either of the following references: Sugarman *et al.*, Ranade, Mayer *et al.*, or Weiner *et al.* The Advisory Action dated November 11, 2004, did not specifically address this rejection. Appellants will assume that the rejection has not been overcome, and will thus respond accordingly.

WO '120 has been cited by the Examiner in the final Office Action to disclose imexon and several of the claimed derivatives for treating cancer, and use of imexon in combination with other anti-cancer agents. The Examiner notes that "what is lacking in WO is the teaching of the use of liposomes as carriers for the delivery of imexon or its derivatives for the treatment of cancer or stimilating the immune system." Final Office Action, paragraph 3, page 6. It is purported that WO '120 teaches the use of slow release carriers. The teachings of Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* are discussed *supra*. According to the Examiner, the use of liposomes as carriers for imexon or its derivatives taught by WO '120 would have been obvious to one of ordinary skill in the art because of the advantages of liposomes taught by Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* The Examiner considered arguments set forth in the response to the previous rejection under 35 U.S.C. §103 (a), and found them unpersuasive, noting that the response set forth above applies to this rejection.

b) *The Examiner's Burden to Establish a Prima Facie Case of Obviousness*

As noted above, in order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *MPEP* § 2142. See also *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). All three elements must be shown to establish a *prima facie* case of obviousness. If one element is missing, a *prima facie* case of obviousness has not been established.

In light of the reasons presented in the previous section, which are specifically incorporated into this section, Appellants disagree that claims 1-2 and 4-32 are obvious over WO '120 in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, or Weiner *et al.* Furthermore, Appellants submit the following additional arguments against the Examiner's assertion of obviousness.

c) *The Examiner has Failed to Establish a Prima Facie Case of Obviousness Because he has Failed to Identify Some Suggestion or Motivation to Combine Reference Teachings*

Appellants contend that there is no *prima facie* case of obviousness because the Examiner has failed to identify some suggestion or motivation to combine reference teachings. None of these references make any suggestion of using liposomes as a carrier of imexon and its derivative. The teachings of Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* are discussed above. Regarding WO '120, as admitted by the Examiner, "what is lacking in WO is the teaching of the use of liposomes as carriers for the delivery of imexon or its derivatives for

the treatment of cancer or stimulating the immune system." Final Office Action, paragraph 3, page 6. Thus, WO '120 makes no mention whatsoever of using liposomes as carriers of imexon or its derivatives.

Therefore, the relevant inquiry is whether one of ordinarily skill in the art, with knowledge that is generally available, would be motivated to combine WO '120 in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.* Appellants contend that one of ordinary skill in the art would not be motivated to combine these references to result in the claimed invention. In accordance with the requirements of *In re Kotzab*, the Examiner has again failed to make any showing of "objective evidence" and "specific factual findings with respect to the motivation to combine references." *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 13113, 1317 (Fed. Cir. 2000). Appellants reiterate that many therapies and drugs are available in treating cancer. Although Sugarman *et al.* provides examples of using liposomes as a carrier of cancer drugs such as doxirubicin and cisplatin, it does not suggest which additional cancer drugs could be delivered using liposomes. As a matter of fact, neither Ranade, Mayer *et al.*, nor Wiener *et al.* makes any recommendation as to potential types of cancer drugs which may warrant future investigation. In view of the myriad of cancer drugs available and the lack of suggestion in the prior art, a skilled artisan would have no motivation to specifically use liposomes as a carrier for imexon and its derivatives. Thus, WO '120, in view of either Sugarman *et al.*, Ranade, Mayer *et al.*, and Weiner *et al.*, fails to establish an element required for a *prima facie* case of obviousness.

d) The Examiner has Failed to Establish a Prima Facie Case of Obviousness Because he has Failed to Identify a Reasonable Expectation of Success to Combine Teachings of the Cited References

Another element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of WO in view of either Sugarman *et al.*,

Ranade, Mayer *et al.*, and Weiner *et al.* would be successful. Appellants contend that there would be no reasonable expectation of success in combining imexon derivatives and liposomal delivery in treating cancer. The Examiner presents no evidence that combining imexon derivatives and liposomes would reasonably result in success. In fact, Sugarman *et al.* states that "liposomal delivery of antitumor therapy is in its ***infancy*** and the optimal liposome/drug formulation has ***not yet been determined.***" Clearly, in view of this statement, one of ordinary skill in the art would understand that using liposomes as carriers of imexon and its derivatives would not reasonably result in success.

Furthermore, combining cancer drug therapies is a highly unpredictable art. Trial and error is often required to determine the proper combination of therapies. For example, Sugarman *et al.* discloses use of liposomes as a carrier for doxorubicin delivered intravenously. In one of the studies, out of 18 patients available for study, only 5 exhibited a ***marginal*** response. Sugarman *et al.*, page 233. These results strongly suggest that success is not even reasonably expected for well-known cancer drugs. As mentioned in the previous section, the prior art, at most, describes an "obvious to try" situation which does not render a claim obvious. Accordingly, the Examiner has not established an element necessary for a *prima facie* case of obviousness.

Further, Presant, cited by the Examiner as of interest, which purportedly teaches that liposomes are also called micelles, fails to provide the missing expectation of success. Presant pertains to methods of targeting locations in a body using micellular particles. It contains no information pertaining to delivery of imexon via liposomal delivery.

e) The Pharmaceutical Compositions Set Forth in the Claims have Exceptionally Superior Activity Against Tumor Cells

As in the prior rejection under 35 U.S.C. §103(a), even if the Examiner had established a *prima facie* case of obviousness, which Appellants strongly assert that this has not been the case, the obviousness rejection could be readily overcome by Appellants' evidence, set forth in the Specification, of the unexpectedly superior activity of certain of the claimed pharmaceutical compositions against tumor cells. See *MPEP* §2144.09, citing *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963); *In re Weichert*, 370 F.2d 927, 152 USPQ 247 (CCPA 1967). The discussion set forth above in subpart 1 is specifically incorporated into this section.

The evidence discussed in detail in subpart 1 pertaining to (1) the superior properties of liposome formulations of particular imexon analogs compared to imexon analog alone; and (2) evidence of the superior potency of imexon analogs against a panel of tumor cells in culture compared to imexon alone, is sufficient to rebut any contention that a *prima facie* case of obviousness has been established by the Examiner.

f) Conclusion

In view of the above arguments, Appellants have shown that the Examiner has not established a *prima facie* case that claims 1-2 and 4-32 were obvious at the time of filing. Furthermore, Appellants have set forth evidence that even if the Examiner had established a *prima facie* case of obviousness, which Appellants assert has not been the case herein, that such a finding could be successfully overcome by the unexpectedly superior properties of the pharmaceutical compositions and imexon derivatives set forth above. Accordingly, Appellants respectfully request that the Board withdraw the rejection of claims 1, 2, and 4-32.

Accordingly, Appellants respectfully request that the Board withdraw the rejection of claims 1, 2, and 4-32.

3. *Claims 1-2 and 4-32 are not obvious over Hermann or WO '120 in view of Presant*

a) *The Examiner's Rejection*

Claims 1-2 and 4-32 are rejected under 35 U.S.C. §103(a) as being unpatentable over Hermann or WO '120, in view of Presant. The teachings of Hermann and WO '120 are discussed above. Presant is said to teach that when micellar particles such as liposomes containing active agents are injected into the host, there is an enhanced retention of the active agent in the tumor cells. The Examiner contends that the use of micellar particles such as liposomes for the delivery of imexon taught by Hermann or WO '120 would have been obvious to one of ordinary skill in the art since Presant shows enhanced accumulation of these particles at the tumor site. The Examiner further notes that "the criticality of the use of phosphatidylcholine and phosphatidylglycerol with a specific fatty acid chain such as myristic acid in specific ratios is not readily apparent in the absence of unexpected results since there are commonly used phospholipids in the preparation of liposomes." Further, it is said that the use of derivatives of imexon would have been obvious to one of ordinary skill in the art since the active skeleton is the cyanozairidine structure.

b) *The Examiner's Burden to Establish a Prima Facie Case of Obviousness*

As discussed in the above 35 U.S.C. §103(a) rejections, in order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *MPEP* §2142. See also *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). All three elements must be shown to

establish a *prima facie* case of obviousness. Thus, if even one element is missing, a *prima facie* case of obviousness does not exist.

c) *The Examiner has Failed to Establish a Prima facie Case of Obviousness Because he has Failed to Identify Some Suggestion or Motivation to Combine Reference Teachings*

As previously noted, one of the elements that is required in order for a *prima facie* case of obviousness to exist is that there must be some motivation or suggestion, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine the teachings of Hermann or WO '120, in view of Presant. "The mere fact that references can be combined or modified does not render the resultant combination obvious ***unless the prior art also suggests the desirability of the combination***." MPEP § 2143.01 (emphasis added). As in the prior 35 U.S.C. §103 (a) rejections discussed *supra*, none of these references makes any suggestion of delivering imexon via administration of liposomes.

Consequently, the issue is whether one of ordinarily skill in the art, with knowledge that is generally available, would be motivated to combine Hermann or WO '120 and Presant. Appellants assert that, as discussed above in the response to the previously addressed 35 U.S.C. §103(a) rejections, no such motivation to combine these references exists. In accordance with the requirements of *In re Kotzab*, the Examiner has once again failed to make any showing of "objective evidence" and "specific factual findings with respect to the motivation to combine references." *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 13113, 1317 (Fed. Cir. 2000).

The Examiner contends that the use of micellar particles such as liposomes for the delivery of imexon taught by Hermann or WO '120 would have been obvious to one of ordinary skill in the art since Presant shows enhanced accumulation of these particles at the tumor site. However, as noted above, hundreds of drugs exist for treating cancer. As discussed above, one

of ordinary skill in the art could not possibly know that imexon would be a drug appropriate for liposomal delivery.

d) The Examiner has Failed to Establish a Prima Facie Case of Obviousness Because he has Failed to Identify a Reasonable Expectation of Success to Combine Teachings of the Cited References

Another element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of WO '120 or Hermann in view of Presant would be successful. As discussed above, Appellants submit that there would be no reasonable expectation of success in combining imexon and liposomal delivery in treating cancer. The Examiner has failed to provide evidence that modifying or combining the teachings of the cited references would reasonably result in success.

Further, as noted above, the result of combining drug therapies is impossible to predict. The Examiner appears to merely assume that combining imexon and liposomal drug delivery will automatically result in success. Once again, there is simply is no basis for such an assumption. Furthermore, none of the cited references provide any guidance or recommendations as to whether combining imexon with liposomal drug delivery would be successful in treating cancer.

In view of the prior art, a person of ordinary skill in the art could not reasonably expect to achieve success in administering imexon via liposomal delivery. As a result, WO '120 or Hermann in view of Presant does not establish a reasonable expectation of success as required for a *prima facie* case of obviousness.

e) The Pharmaceutical Compositions Set Forth in the Claims have Exceptionally Superior Activity Against Tumor Cells

As in the prior rejection under 35 U.S.C. §103(a), even if the Examiner had established a *prima facie* case of obviousness, which Appellants strongly assert that this has not been the case,

the obviousness rejection could be readily overcome by Appellants' evidence, set forth in the Specification, of the unexpectedly superior activity of certain of the claimed pharmaceutical compositions against tumor cells. See *MPEP* §2144.09, citing *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963); *In re Weichert*, 370 F.2d 927, 152 USPQ 247 (CCPA 1967). The discussion set forth above in subpart 1 is specifically incorporated into this section.

The evidence discussed in detail in subpart 1 pertaining to (1) the superior properties of liposome formulations of particular imexon analogs compared to imexon analog alone; and (2) evidence of the superior potency of imexon analogs against a panel of tumor cells in culture compared to imexon alone, is sufficient to rebut any contention that a *prima facie* case of obviousness has been established by the Examiner.

f) Conclusion

In view of the above arguments, Appellants have shown that the Examiner has not established a *prima facie* case that claims 1-2 and 4-32 were obvious at the time of filing. Furthermore, Appellants have set forth evidence that even if the Examiner had established a *prima facie* case of obviousness, which Appellants assert has not been the case herein, that such a finding could be successfully overcome by the unexpectedly superior properties of the pharmaceutical compositions and imexon derivatives set forth above. Accordingly, Appellants respectfully request that the Board withdraw the rejection of claims 1, 2, and 4-32.

Accordingly, Appellants respectfully request that the Board withdraw the rejection of claims 1, 2, and 4-32.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, none of the pending claims are properly rejected under 35 U.S.C. §103. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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Date: December 30, 2005

APPENDIX 1

CLAIMS APPENDIX

1. A pharmaceutical composition, comprising an imexon or derivative thereof in combination with one or more phospholipids.

2. The pharmaceutical composition of claim 1, wherein at least a portion of the phospholipids comprise micelles.

4. The pharmaceutical composition of claim 3, wherein a portion of the phospholipids comprise liposomes.

5. The pharmaceutical composition of claim 1, wherein the phospholipid is dimyristoyl phosphatidyl choline, dimyristoylphosphatidylglycerol or phosphatidic acid.

6. The pharmaceutical composition of claim 1, comprising a plurality of phospholipids.

7. The pharmaceutical composition of claim 6, wherein the phospholipids comprise dimyristoyl phosphatidyl choline and dimyristoylphosphatidylglycerol.

8. The pharmaceutical composition of claim 7, further defined as comprising dimyristoyl phosphatidyl choline and dimyristoylphosphatidylglycerol in a 7:3 molar ratio.

9. The pharmaceutical composition of claim 6, wherein the plurality of lipids comprise dimyristoyl phosphatidyl choline and phosphatidic acid.

10. The pharmaceutical composition of claim 9, further defined as comprising dimyristoyl phosphatidyl choline and phosphatidic acid in a 7:1 molar ratio.

11. The pharmaceutical composition of claim 1, wherein the imexon derivative thereof is hydrophobic.

12. The pharmaceutical composition of claim 1, wherein composition comprises imexon.

13. The pharmaceutical composition of claim 1, wherein the composition comprises at least one derivative of imexon.

14. The pharmaceutical composition of claim 13, wherein the derivative of imexon comprises 2-cyanoaziridine-1-(N-benzyl) carboxamide.

15. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-(N-benzyl) carboxamide.

16. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(3,4-dichlorophenyl)] carboxamide.

17. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(4-fluorophenyl)] carboxamide.

18. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(4-trifluorophenyl)] carboxamide.

19. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(4-ethoxycarbonylphenyl)] carboxamide.

20. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(ethoxycarbonyl)methyl] carboxamide.

21. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(3-pyridyl)] carboxamide.

22. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(1-naphthyl)] carboxamide.

23. The pharmaceutical composition of claim 11, wherein the derivative of imexon comprises 2-cyanoaziridine-1-[N-(3-acetylphenyl)] carboxamide.

24. The pharmaceutical composition of claim 13, wherein the derivative of imexon comprises 2-cyanoaziridine-1-(N-methyl) carboxamide, 2-cyanoaziridine-1-[N-carbonic dichloride] carboxamide, 2-cyanoaziridine-1-(N-ethyl)carboxamide,

2-cyanoaziridine-1-[N-(2,4-dichlorophenyl)] carboxamide, 2-cyanoaziridine-1-[N-(3,4-dichlorophenyl)] carboxamide, 2-cyanoaziridine-1-carboxamide, 2-cyanoaziridine-1-(N-*t*-butyl) carboxamide, 2-cyanoaziridine-1-(N-phenyl) carboxamide, 2-cyanoaziridine-1-(N-cyclohexyl) carboxamide, 2-cyanoaziridine-1-(N-butyl) carboxamide, 2-cyanoaziridine-1-[N-(*p*-nitrophenyl)] carboxamide, 2-cyanoaziridine-1-[N-(*bis*-cyanoaziridine)] carboxamide, 2-cyanoaziridine-1-[N-(4-ethoxycarbonylphenyl)] carboxamide, 2-cyanoaziridine-1-[N-(ethoxycarbonyl)methyl] carboxamide, 2-cyanoaziridine-1-[N-(3-pyridyl)] carboxamide, 2-cyanoaziridine-1-[N-(4-sulfamylphenyl)] carboxamide, 2-cyanoaziridine-1-[N-(1-naphthyl)]carboxamide, 2-cyanoaziridine-1-[N-(2-acetoxyphenyl)] carboxamide or 2-cyanoaziridine-1-[N-(3-acetylphenyl)] carboxamide.

25. The pharmaceutical composition of claim 1, further comprising a targeting agent, a diagnostic agent or a second therapeutic agent.

26. The pharmaceutical composition of claim 25, wherein said targeting agent, diagnostic agent or second therapeutic agent is covalently attached to said phospholipids by a linking moiety.

27. The pharmaceutical composition of claim 25, comprising a second therapeutic agent.

28. The pharmaceutical composition of claim 26, wherein said second therapeutic agent comprises an anticancer agent.

29. The pharmaceutical composition of claim 28, wherein the anticancer agent is chemotherapy agent, a radiotherapy agent, an immune therapy agent, a genetic therapy agent, a hormonal therapy agent or a biological agent.

30. A pharmaceutical liposome composition, comprising an imexon or a derivative thereof in combination with one or more phospholipids.

31. A method of treating an individual with cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising an imexon or derivative thereof in combination with one or more phospholipids.

32. A method of stimulating the immune system of an individual, comprising administering a therapeutically effective amount of a composition comprising an imexon or derivative thereof in combination with one or more phospholipids.

APPENDIX 2

EVIDENCE APPENDIX

Exhibit 1: Hermann (U.S. Patent No. 5,369,119); cited in Office Action Dated November 14, 2003

Exhibit 2: Sugarman *et al.*, (*Critical Reviews in Oncology Hematology*, (1992)); cited in Office Action Dated November 14, 2003

Exhibit 3: Ranade, (*J. Clin. Pharmacol.*, (1989)); cited in Office Action Dated November 14, 2003

Exhibit 4: Mayer *et al.*, (*Cancer Letters*, (1990)); cited in Office Action Dated November 14, 2003

Exhibit 5: Weiner *et al.*, (*Drug Development and Industrial Pharmacy*, (1989)); cited in Office Action Dated November 14, 2003

Exhibit 6: WO 99/00120; cited in Office Action Dated November 14, 2003

Exhibit 7: Presant *et al.* (U.S. Patent No. 5,435,989); cited in Final Office Action Dated July 14, 2004

APPENDIX 3

RELATED PROCEEDINGS APPENDIX

None.

Critical Reviews in Oncology/Hematology, 1992; 12:231-242

ONCHEM 024

Liposomes in the treatment of malignancy: a clinical perspective

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(Accepted 30 January 1992)

Contents

Introduction

Since Bangham's original description of bilayered ıospholipid vesicles in 1965, liposomes have received uch attention as transporters of pharmacological ;ents [1]. These vesicles, ranging in size from 0.025 icrons to greater than 20 microns, are composed of ıgle or multiple phospholipid membranes surround- an aqueous compartment. According to their hydrophilic or hydrophobic tendencies, drugs can be entrapped in the aqueous or membrane phases, respectively. The preclinical evaluation of their use in the treatment of malignant disease has generated a considerable body of literature. However, the complexities of mass producing standard formulations for clinical trials has hampered their use in humans until the past several years.

Human trials of anti-cancer agents formulated in li-

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posomes have either employed liposomes as carriers of chemotherapeutic agents or biologic response modifiers. Most of the chemotherapeutic agents used have been doxorubicin or cisplatin derivatives [2–6]. Muramyl peptides have been used as macrophage activators to invoke antitumor biologic responses in the host [7–9].

Phase I and II trials using liposomes in cancer patients consistently demonstrate their safety. Encapsulation is often associated with a favorable effect on the therapeutic index, which is usually attributable to a reduction in toxicity of the active compound. Low-grade fever and mild fatigue are the most common toxicities related specifically to the lipid constituents. Although objective tumor responses and stable disease during treatment have been observed in early studies, further clinical trials are required to define the role of liposomal antitumor therapy in medical oncology.

II. Rationale

The incorporation of drugs into liposomes has several theoretical advantages. They protect their contents from interaction with plasma components, while favorably altering the pharmacokinetics and biodistribution of the free compound. For example, preclinical data demonstrated that doxorubicin encapsulated in liposomes was significantly less cardiotoxic than the free compound [11–14], and the lower toxicity correlated with lower cardiac levels of the drug. The reduced toxicity may be associated with a slower release of doxorubicin, since it has been shown that continuous infusion doxorubicin is less cardiotoxic to humans [15]. Since liposomes do not readily penetrate biologic membranes, they can be used for the controlled release of drugs within body cavities such as the pleural, peritoneal or intrathecal spaces. Khatibi has shown that when cytarabine encapsulated in multilamellar vesicles is administered intrathecally in rhesus monkeys, cytotoxic levels could be maintained for 672 h [16]. Similar studies have demonstrated the efficacy of the intraperitoneal administration of liposomal doxorubicin [5].

Lipophilic agents that are either incompatible with intravenous injection or require large volumes of solvent can be administered safely when entrapped in liposomes. As discussed subsequently, NSC 251635, a water-insoluble compound, has been rendered biocompatible by incorporation into a liposome membrane [17, 18].

Although simple phospholipid membranes will naturally target to the reticuloendothelial system (RES), manipulations of the liposome surface can be used for organ-specific or tumor-specific targeting. Gabizon et al. have demonstrated that tumor localization can be achieved through the use of small unilamellar vesicles containing a small fraction of monosialoganglioside or hydrogenated phosphatidylinositol [19]. Holmberg et al., using organ-targeted therapy, have administered liposomes coated with antibodies specific for pulmonary endothelium to treat lung metastases in mice [20]. Others have added tumor-specific antibodies to the liposome surface to target therapy [21–23].

III. Pharmacologic considerations

By altering their physical parameters such as size, electrostatic charge, phospholipid profile, and membrane characteristics, liposomes can be engineered to efficiently encapsulate different types of drugs and effectively transport them within the circulation. Each drug encapsulated in a liposome must be regarded as a unique pharmacologic entity dependent upon the components of the liposome and the conditions under which drug encapsulation takes place. Small changes in liposomal preparation or composition can have profound effects on drug bioavailability, activity, and toxicity [24, 25].

Since liposomes produced by standard methods are sequestered primarily by the RES, they can be used to target therapy directly to malignant disease of the liver and spleen or activate macrophages for immunomodulation. By manipulating the physical properties of vesicles, for example, using small unilamellar vesicles composed of uncharged lipids, the RES can be avoided, circulation time increased, and tumor-targeting augmented.

There are two types of commonly used liposomes: multilamellar vesicles (MLVs; 1–5 μm) and unilamellar vesicles (UVs; 0.05–0.2 μm [26–28]. Because MLVs are composed of concentric layers of phospholipids, the aqueous compartment is reduced, allowing for better encapsulation of lipophilic drugs. These drugs associate with the inner and outer phospholipid membranes. When MLVs are subjected to sonication or extrusion through filters, UVs are formed. Small UVs (SUVs) measure less than 0.1 μm; large UVs (LUVs) measure between 0.1 and 0.25 μm. Generally, UVs are better suited for delivery of hydrophilic drugs.

Since large liposomes are rapidly sequestered by the RES, SUVs are employed to increase liposome circulation time [29]. In general, the smaller the vesicle, the longer the circulation time. Small vesicles are utilized for slow drug release in the circulation and for non-RES targeting [19].

IV. Liposomal delivery of chemotherapeutic agents

IV-A. Intravenous doxorubicin

Doxorubicin has a wide spectrum of clinical activity that includes the treatment of lymphoma, myeloma, sarcoma, ovarian carcinoma, breast cancer, and other solid tumors. Its dose-limiting toxicity is myocardial damage although mucositis, stomatitis, and myelosupression are other well-recognized side effects. Administering doxorubicin by slow infusion in humans [15] or by liposomal encapsulation in animals [11–14] reduces cardiac toxicity and allows higher total doses to be tolerated. Animal data also suggests that liposomal doxorubicin is less myelosupressive than the free drug and is less likely to cause soft-tissue necrosis [30, 31].

Gabizon et al. reported a phase I clinical trial in which liposome-encapsulated doxorubicin (LED) was used to treat 32 patients with metastatic cancer after failure of conventional chemotherapy [2]. The liposomes were composed of phosphatidylcholine (PC), phosphatidylglycerol (PG), Cholesterol (Chol), and D-α-tocopherol succinate in a molar ratio of 7:3:4:0.2, respectively. The mean size was in the range of 0.3–0.5 μm (Table 1). Liposomes were infused at a concentration of 0.5–2.0 mg ml, at a rate of 2–3 ml/min through a peripheral vein. Twenty-seven patients had primary or secondary hepatic involvement. Primary tumors were predominantly colorectal (50%) and hepatocellular (28%). Patient characteristics are listed in Table 2. Interestingly, gastrointestinal toxicity was mild without the use of prophylactic antiemetics. Fever was the most common toxicity (37%), whereas stomatitis and myelosupression were the dose-limiting toxicities. The maximum tolerated dose (MTD) was 120 mg/m^2 (Table 3).

Of the 18 patients evaluable for antitumor effect, one partial response and five marginal responses were observed. The duration of response was only 3 to 8 months. All responders had primary or metastatic hepatic involvement; two patients had been previously treated with intravenous doxorubicin. Two patients with colorectal disease metastatic to liver had stable disease over a 2 to 4 month period. Based on these results, Gabizon concluded that liposomal doxorubicin can be safely administered at higher dose levels than the free drug and that phase II studies starting at a dose of 100 mg/m^2 are indicated.

Rahman et al. performed a phase I study using LED in the treatment of 14 patients with malignancies for which no effective therapy exists [3]. Three patients had adenocarcinomas of unknown primary, three patients had lung primaries, and eight had either a primary myeloma, colon carcinoma, melanoma, breast carcinoma, pancreatic cancer, small cell colon cancer, hemangiosarcoma or cystosarcoma phylloides. Six patients received previous combination chemotherapy,

TABLE 1

Liposome preparation

Agent	Manufacturer	Lipid Composition	Type	Size	Lipid/Drug Ratio
Doxorubicin	Hadassah Medical Center	PC/PG/Chol/D-α-t 7:3:4:0.2	Small vesicle	0.3–0.5 μm	5–120 μmol: 1 mg
Doxorubicin	Georgetown Univ. Medical Center	CL/PC/Chol/SA	LUVs	0.9–1.2 μm	12 mg: 1 mg
NDDP	M.D. Anderson Cancer Center	DMPC/DMPG 7:3	MLVs	1–5 μm	15 mg: 1 mg
NSC 251635	Institut Jules Bordet (Brussels)	PC/Chol/SA	SUVs	30–110 nm	20 mg: 343 μg: 1 mg
MTP-PE	CIBA-CEIGY	PC/PS 3:1	MLVs	1–5 μm	250 mg: 1 mg
DTR-GDP	Univ. N.D. Medical School	PC/PG 7:3	MLVs	3.01±0.31 μm	250 mg: 1 mg

Abbreviations. PC, phosphatidylcholine; PG, phosphatidylglycerol; chol, choesterol; D-α-t, D-α-tocopherol succinate; CL, cardiolipin; SA, stearylamine; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; PS, phosphatidylserine; NDDP, *cis*-bisneodecanoato-*trans*-*R,R*-1,2-diaminocyclohexane platinum (II); MTP-PE, muramyl tripeptide phosphatidylethamolamine; DTR-GDP, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanylglyceryl dipalmitate; SUVs, small unilamellar vesicles; MLVs, multilamellar vesicles.

TABLE 2

Patient characteristics

Primary investigator	N	Primary tumor	Performance status	Drug	Schedule
Gabizon	32	mixed solid	Karnofsky 70%	Doxorubicin	20–120 mg/m² i.v. q 3 weeks
Rahman	14	mixed	ECOG 0-1	Doxorubicin	30–90 mg/m² i.v. q 3 weeks
Treat	20	breast	ECOG 0-3	Doxorubicin	75 mg/m² i.v. q 3 weeks
Delgado	15	ovarian	ECOG 0-2	Doxorubicin	20–100 mg/m² i.p. q 3–4 weeks
Seaber	14	mixed	Karnofsky 20–100%	NSC 251635	82–456 mg/m² i.v. q 7 to 50 days
Perez-Soler	39	mixed	ECOG 0-2	NDDP	7.5–390 mg/m² i.v. q 4 weeks
Murray	28	mixed solid	Zubrod 0-2	MTP-PE	0.05–12 mg/m² i.v. biw × 9 weeks
Urba	27	mixed solid	ECOG 0-1	MTP-PE	0.1–2.7 mg/m² i.v. q wk × 8 weeks
Creaven	37	mixed solid	ECOG 0-2	MTP-PE	0.01–6.0 mg/m² i.v. b.i.w. × 4 weeks
Vosika	12	mixed solid	Karnofsky ≥60%	DTP-GDP	0.2–1.2 mg/m² i.v. q week
Phillips	13	melanoma	ECOG 0-2	TAA	35–175 mg s.c. q 2–4 weeks

one patient received only radiation therapy and seven were previously untreated. Patients with a history of cardiac disease or prior anthracycline exposure were excluded from study.

Liposomes composed of 14 mg of lipid for each 1 mg of doxorubicin were 0.9–1.2 μm in diameter. Treatment at a concentration of 1 mg/ml was administered over 10–45 min with escalating doses of LED, from 30–90 mg/m², every 21 days.

Although the dose limiting-toxicity was granulocytopenia, which occurred after doses of 60 mg/m² (four patients) and 90 mg/m² (five patients), nonlimiting thrombocytopenia and normochromic, normocytic anemia were noted as well. Granulocyte nadirs of less than 200 cells/ml were seen 10–15 days after treatment and in most cases resolved within 10 days. Illustrating the importance of liposomal preparation in the determination of biologic effects, Rahman in contrast to Gabizon, noted the absence of stomatitis. Alopecia was seen in all five patients at the 90 mg/m² dose level; however, gastrointestinal toxicity was limited to grade 1 and 2 nausea and vomiting. No cardiac toxicity was noted, even though one patient received a cumulative dose of 885 mg/m². Two idiosyncratic events were the development of chills in one patient and lower back pain in another. None of the patients developed venous phlebitis after infusion of LED. No end organ toxicity was noted.

Pharmacokinetics studies revealed at $t_{1/2}$ $-\alpha$ of 4–6 min and a $t_{1/2}$ $-\beta$ of, in general, 350–450 min. Doxorubicin metabolites appeared in plasma at low levels after 12–24 h. Urinary excretion of doxorubicin following 60 or 90 mg/m² was 10% after 24 h. The major route of excretion was presumed to be biliary.

No objective tumor responses were seen in this unfavorable cohort of patients. However, because LED was less toxic than free doxorubicin, showed a slower conversion to metabolites, and had a favorable area under the curve, the author felt that phase II trials were indicated, especially in breast cancer where LED had demonstrated objective responses.

Treat et al. evaluated the antitumor activity of LED in 20 patients with advanced breast cancer in a phase II study [4]. Patients receiving anthracyclines for metastatic disease or as adjuvant therapy more than 1 year prior to entry were eligible for study. All patients had metastatic disease primarily to the lung (50%), bone (45%), liver (35%) and pleura (35%). Radionuclide ventriculograms in all patients at approximately 350 mg/m² and 550 mg/m², and endomyocardial biopsies in selected patients were performed. Twelve patients had been previously treated with cyclophosphamide, methotrexate and fluorouracil (CMF), four with radiation and CMF, one with radiation alone, and one with elliptinium. Liposomes were prepared as described by Rahman [30].

Patients received an LED dose of 75 mg/m² by slow intravenous infusion; however, the dose was reduced if the patient received prior radiation therapy or had extensive hepatic metastases. The cumulative dose ranged from 120 to 880 mg/m² with eight patients receiving more than 580 mg/m² of doxorubicin. Hematologic toxicity was mild, with an overall mean granulocyte nadir of 3740/μl and mean platelet nadir of 223000/μl. No patient developed sepsis, systemic infection, or a bleeding complication. Only two patients required dose reduction for myelosupression. Other toxicities included alopecia (100%), mild gastrointestinal symptoms (30%), grade 4 vomiting (10%), and mild stomatitis (10%). Of the eight patients receiving more than 500 mg/m² of doxorubicin, five endomyocardial biopsies were performed. Four were normal; the other showed mild disease (Billingham grade 1 toxicity).

Nine of 20 patients demonstrated a response, five of which were complete responses in the index lesions. The duration of response was 3–12 months, with a mean of

7. Two of the responders had previously received doxorubicin in combination with cyclophosphamide and fluorouracil. These data are encouraging because they demonstrate that cardiac and gastrointestinal toxicity is minimized when encapsulated doxorubicin is used. This allows for more effective dosing, i.e., a greater area under the curve, and achieves a favorable response rate (45%) in previously treated patients.

Recently, a phase I/II trial of LED in the treatment of non-small cell lung cancer was reported [32]. TLC D-99, a liposomal doxorubicin preparation (The Liposome Company, Princeton, NJ), was used to treat 14 patients with nonresectable disease (stage IIIB, IV). The dose-limiting toxicity was grade 4 granulocytopenia at 75 mg/m^2. Other toxicities included thrombocytopenia, mucositis, chills, fever, sepsis and back pain. One patient had a partial response lasting 4 weeks; one patient had a minor response.

Excluding cardiotoxicity, toxicities in all of the clinical trials utilizing intravenously administered LED parallel those of free doxorubicin (Table 3). Myelosupression was dose-limiting and all investigators noted a significant incidence of nausea, vomiting and alopecia. Interestingly, while Treat and Gabizon found stomatitis to be dose-limiting in a subset of patients, Rahman did not report a significant incidence of stomatitis. Since Treat and Rahman used the same preparation and dosing schedules, the reason for this discrepancy is not clear. Gabizon is the only investigator to report a significant incidence of fever; however, it is not clear whether this is related to the liposome preparation. None of the investigators reported cardiotoxicity as a significant side-effect of LED.

IV-B. Intraperitoneal doxorubicin

A phase I/II study evaluating the treatment of advanced ovarian cancer with intraperitoneal administration of LED has been reported [5]. Although free doxorubicin is effective in the treatment of ovarian cancer when administered intravenously, treatment failure is often attributed to residual peritoneal disease, which is less amenable to intravascular therapy [33, 34]. Intraperitoneal delivery of free doxorubicin has been limited by severe peritonitis. Delgado evaluated the use of intraperitoneal LED in order to ameliorate the dose-limiting peritonitis associated with the free drug.

Fifteen patients with advanced (stage III and IV) ovarian carcinoma were treated every 3 or 4 weeks with 20–100 mg of LED diluted in 2 liter of normal saline that was delivered through an Infusaport and drained after 4 h of infusion. All patients had received prior intravenous chemotherapy, eight had received prior doxorubicin.

The drug was very well tolerated, with minimal hematologic toxicity. One patient developed chemical peritonitis that occurred after a 60 mg dose. The lowest nadir white cell count was 2700 cells/μl. The lowest

TABLE 3

Maximum tolerated dose (MTD) and toxicity

Agent	Investigator	MTD	Dose-limiting toxicity	Other toxicities
Doxorubicin (Hadassah)	Gabizon	120 mg/m^2	myelosuppression, stomatitis	fever, chills, nausea, vomiting, alopecia; rare: hypotension
Doxorubicin (Georgetown)	Rahman	90 mg/m^2	myelosuppression	alopecia, nausea, vomiting; rare: chills, back pain
Doxorubicin (Georgetown)	Treat	75 mg/m^2	myelosuppression	alopecia, nausea, vomiting, stomatitis
Doxorubicin (Georgetown)	Delgado	100 mg/m^2 (i.p.)	myelosuppression	abdominal pain, diarrhea, nausea, vomiting; rare: granulocytopenia
NDDP (M D. Anderson)	Perez-Soler	312.5 mg/m^2	myelosuppression	nausea, vomiting, fever, diarrhea, fatigue, transaminitis; rare: neuropathy
NSC 251635 (Brussels)	Sculier	4.56 mg/m^2	NR	drowsiness, back pain, fever, chill; rare: bronchospasm, rash, respiratory distress
MTP-PE (CIBA-Ceigy)	Murray	6.0 mg/m^2	Malaise	chills, fever, malaise, nausea, vomiting, headache, anorexia, myalgia, hypertension, hypotension dyspnea, tachycardia, rare diaphoresis, vertigo, cough
MTP-PE	Urba	2.7 mg/m^2	NR	chills, fever, nausea, hypotension, fatigue
MTP-PE	Creaven	6.0 mg/m^2	NR	chills, fever, hypertension
MTP-GDP	Vosika	1.2 mg/m^2	NR	chills, fever, nausea, hypotension

Abbreviation: NR, not reached.

platelet count was 171 000/μl. Nausea and vomiting were rare, with only four patients requiring intravenous hydration. No patient developed mucositis or alopecia. Three of four patients with minimal disease at the initiation of therapy demonstrated a surgical partial response. None of the six patients with bulky disease demonstrated a significant response; however, three of them noted an improvement in their ascites. Pharmacokinetics evaluation revealed a greater than 50-fold advantage for intraperitoneal drug level area under the curve compared with that of the plasma. Although dose-limiting toxicity was not reached in this study, intraperitoneal administration of LED has been demonstrated to be safe and effective in patients with ovarian cancer who have minimal residual disease after previous therapy.

IV-C. Lipophilic platinum-containing compounds

Cisplatin has a broad spectrum of activity in solid tumors and some lymphomas. Its main toxicities include nephrotoxicity and neurotoxicity. A lipophilic derivative of cisplatin, *cis*-bis-neodecanoato-*trans-(R,R)*-1,2-diaminocyclohexaneplatinum(II) (NDDP), was developed at M.D. Anderson Cancer Center for liposomal incorporation [6]. The large MLVs were formulated with dimyristoylphosphatidylcholine and dimyristoylphosphatidyl-glycerol in a ratio of 7:3. The dose-limiting toxicity of liposomal NDDP (L-NDDP) in mice and dogs is myelosupression and a gastrointestinal hemorrhagic syndrome, respectively [35–36]. Nephrotoxicity was not seen. L-NDDP was found to be more active than cisplatin in the treatment of liver and spleen metastases in a mouse reticulosarcoma model [37].

Perez-Soler investigated the use of L-NDDP in a phase I study involving 39 patients with a variety of tumors including colon (18), lung (4), head and neck (4), renal cell (3), sarcoma (3), germ cell (2) and one each with lymphoma, melanoma, breast, islet cell and gastric carcinoma. All patients were considered refractory to conventional chemotherapy; 20 patients had previously been treated with cisplatin. L-NDDP was given intravenously once every 4 weeks in the inpatient service. The starting dose was 7.5 mg/m^2, on a dose escalation schedule of upto 390 mg NDDP/m^2. The rate of all infusions was 4 mg NDDP/min.

The most common toxicities were gastrointestinal, with dose-related nausea and vomiting starting at 15 mg/m^2 and diarrhea at 100 mg/m^2. Fever, not requiring treatment, occurred 10–12 h after infusion in most patients receiving more than 100 mg/m^2 L-NDDP. Myelosupression of all hematopoietic precursors was seen at doses higher than 200 mg/m^2. Granulocytopenia was dose dependent with a mean nadir count of 500 cells/μl occurring 12 days after a dose of 390 mg/m^2. No episodes of infection or hemorrhage were observed. Malaise and a mild reversible elevation of alanine aminotransferase were seen at 390 mg/m^2. Two patients complained of mild parasthesias of the hands and feet. Interestingly, there was no creatinine elevation at any dose. Of the 33 evaluable patients, nine remained stable and 23 had disease progression. Of the stable patients, two had a marginal response. Overall, the toxicity profile was more similar to carboplatinum than to *cis*-platinum. A phase II trial at M.D. Anderson Hospital is presently underway to determine the therapeutic efficacy of L-NDDP.

IV-D. Other investigational water-insoluble agents

Because of their hydrophobic properties, water-insoluble drugs cannot be administered intravenously. Hydrophobic drugs, however, can be incorporated into the liposomal membrane to permit intravascular delivery. NSC 251635, a water-insoluble cytostatic derivative of quinazolone, was incorporated into liposomes (L-NSC) and evaluated for clinical efficacy in a pilot study reported in 1983 and updated in 1985 [17, 18]. Sculier et al. entrapped NSC 251635 in the membranes of 30–110 nm vesicles containing phosphatidylcholine, cholesterol and stearylamine in a 4:3:1 ratio.

Fourteen patients (Table 1) with progressive malignancy refractory to conventional therapy were treated. Most patients studied had non-small-cell lung cancer (9), although patients with head and neck cancer (2), adenocarcinoma of unknown origin (2), and erythroleukemia (1) were evaluated. All patients had received previous treatment; two patients had been treated with radiation alone, three with chemotherapy, and nine with both modalities. Eighty-two to 456 mg/m^2 of NSC 251635 in 1 liter fluid was administered at a rate of 100–500 ml/h in the medical intensive care unit.

Toxicity was mild with transient drowsiness, the most common symptom, occurring in five patients. Acute lumbar pain was observed in three patients whose infusion rate was greater than 500 ml/h, but was controlled by slowing the infusion rate. Three patients developed culture-negative fever and chills. Acute respiratory distress occurred during liposome infusion in one patient who also had superior vena caval syndrome. It was surmised that the patient had a pulmonary embolism that may have been related to the infusion. Rash occurred in one patient. Of questionable clinical importance was the observation of activation of the complement system, as evidenced by a decrease in the total hemolytic complement activity (CH'50). No chronic to-

xicities were noted clinically or at autopsy. Pharmacokinetics suggested a two compartment model of drug distribution and implied early dissociation of drug from liposomes. Interestingly, plasma levels of NSC 251635 were detected 120 h after administration.

Three patients with non-small-cell lung cancer did not show evidence of tumor progression. Hence, there was no objective regression of tumor during treatment. It should be noted that these patients were not expected to respond to any standard chemotherapeutic agent, since most had tumors that were known to be refractory de novo and all of them had been previously treated. Sculier did demonstrate that high volumes of lipids could be administered safely and that liposomes provide an appropriate vehicle for the delivery of water-insoluble agents.

V Liposomal delivery of immunomodulators

V-A. *Intravenous muramyl peptides*

Extensive preclinical data in animal tumor models support the use of liposomes as carriers of immunomodulators. Muramyl dipeptides (MDPs) are structurally related to bacterial cell wall constituents and are the smallest component of the Bacillus Calmette-Guerin membrane that invokes an immune response. Muramyl tripeptide phosphatidylethanolamine (MTP-PE), a related compound, can easily be incorporated into liposomal membranes and has been shown to have antineoplastic properties attributed to monocyte activation [38, 39]. MTP-PE has been successful in eradicating spontaneous lung and lymph node metastases in mice inoculated with B-16/BL6 melanoma.

A phase I clinical trial of liposomal MTP-PE (L-MTP-PE) was performed by Murray et al. in 1989 [7]. Twenty-eight patients with various tumors were entered, 24 of whom were subsequently evaluable. Their tumors were colorectal (10), melanoma (1), renal cell (5), lung (1), breast (1), stomach (1), sarcoma (3), salivary gland (1), undifferentiated (1). Twenty patients had received previous chemotherapy, 19 radiotherapy, and 12 immunotherapy. Only one patient had not received previous treatment.

Synthetic liposomes containing a 7:3 molar ratio of phosphatidylcholine to phosphatidylserine were manufactured by CIBA-GEIGY (Basel, Switzerland). The phospholipid to MTP-PE ratio of the final product was 250:1. Patients received escalating doses of 0.05 mg/m^2 to 12.0 mg/m^2 MTP-PE twice weekly for a total duration of 9 weeks. Those with stable disease or responding to treatment could continue the drug beyond 9 weeks.

Constitutional symptoms including chills, fever, malaise, nausea/vomiting, headache, anorexia and myalgias were seen in most patients, and all appeared to be dose dependent. Hypertension, hypotension, diaphoresis, tachypnea and/or tachycardia were seen in more than one third of patients evaluated. Rarer complications included diarrhea, vertigo and cough. Interestingly, side effects were more common with the first treatment than with subsequent therapy. No evidence of cumulative toxicity or end-organ damage was apparent. Grade III malaise, the dose-limiting toxicity, was seen in all patients receiving more than 6.0 mg/m^2 of L-MTP-PE. The only significant serum chemistry change was a reduction in serum cholesterol by approximately 8%.

Although the absolute monocyte count did not change during treatment, a significant increase in the absolute granulocyte count was observed at doses of 2 mg/m^2 or higher. The total lymphocyte count was depressed at doses of 4 mg/m^2; however, absolute numbers of lymphocyte subpopulations were unchanged. Acute-phase reactants including c-reactive protein (CRP), β_2-microglobulin, and ceruloplasmin were significantly elevated. Monocyte tumoricidal activity (MTA) was assayed by measuring tumor viability after coculturing patient monocytes with a human melanoma cell line. Patients with low baseline MTA had a significant increase in MTA after treatment, whereas those with high baseline MTA had an actual reduction in MTA. Skin test response to recall antigens did not change significantly during treatment.

There were no objective tumor responses. Three patients had no evidence of tumor progression for a mean duration of 17 weeks. The lack of response was not surprising in this group of patients with refractory disease. Also, animal data suggested that L-MTP-PE is only effective when there is minimal tumor burden [40, 41]. Because of a lack of dose-related tumor response and significant toxicity at higher doses, Murray recommends that L-MTP-PE be at 2.0 mg/m^2 in future trials evaluating patients with less tumor burden.

In May of 1990, Urba et al. reported the use of MTP-PE (CIBA-GEIGY Ltd.) in 27 patients with advanced malignancies [8]. Primaries Tumors included colorectal (15), melanoma (4), renal cell (4), lung (1), rectal (1), ovarian (1) and adenoid cystic of the trachea (1). Most patients had received prior therapy with radiation (10), immunotherapy (12) or chemotherapy (8), either alone or in combination; however, five patients had been previously untreated. Twenty-one of the 25 evaluable patients received all 8 weeks of treatment. Patients received 0.1–2.7 mg/m^2 MTP-PE intravenously, over 1 h, once weekly for a total of 8 weeks.

Most patients experienced chills and fevers. Twelve

patients experienced grade I hypotension, and three required medical intervention for transient decreases in blood pressure of 20–20 mm Hg (grade II) that appeared to be dose-related. Several patients suffered transient fatigue, but no patient had a reduction in performance status during the treatment period. Notably, there were no hematologic side effects observed.

Whereas no objective responses were noted, 11 patients showed no evidence of progression of disease during treatment. Three of these patients exhibited clinical improvement, minor tumor regressions, or both. Immunologic monitoring revealed increased neopterin and c-reactive protein levels that did not appear to be dose-related. There was a consistent decrease in monocyte Fc receptor concentration at 6 h, which returned o baseline at 24 h. There was no consistent change in -interferon levels, class II MHC concentration or D14 expression. In summary, Urba has demonstrated consistent biologic response after injection of MTP-E with little clinical antitumor response. Since the ob-rved biologic response was not dose related and an ITD was not reached, the optimal dosing schedule mains to be determined. Combining MTP-PE with her biologic therapy such as γ-interferon has been ggested to augment tumoricidal activity [42, 43]. Since yelosupression was not an observed toxicity, future mbinations of MTP-PE with chemotherapy would be rational approach to the treatment of advanced ma-nancy.

Creaven et al. [9] using a similar MTP-PE prepara-n. treated 37 patients with advanced cancer [9]. The dominant tumors studied were renal cell cancer (13) d melanoma (6), although other solid tumors were resented: non-small-cell lung cancer (4), colorectal ncer (3), soft-tissue sarcoma (3), unknown primary , and six with miscellaneous tumors. Doses of 0.01– mg/m² were administered intravenously over 1 h ce/weekly for 8 weeks. The drug could be continued ond 8 weeks if the patient demonstrated a clinical ponse or stabilization of disease.

n agreement with Murray, Creaven noted fever, rs, tachycardia, hypertension and tachypnea as the dominant toxicities. The toxicity was usually test with the first dose, and although there was a d, no clear dose-dependent toxicity was noted. No ificant changes in end-organ function, hematologic, lood clotting parameters were detected. Although macrophage stimulation assay results were not com-ly consistent, doses of 0.8–1.2 mg/m² produced an edictable response, whereas a stimulatory effect recorded at 4 mg/m². Paradoxically, the 6.0 mg/m² inhibited macrophage activation. The response was limited to one patient with renal cell carcinoma who had complete resolution of pulmonary metastases lasting 3 months after three courses at a dose of 1.2 mg/m². After relapse, the tumor was unresponsive to L-MTP-PE.

Vosika et al. reported the results of a phase I trial of a liposome-bound lipophilic disaccharide tripeptide used to treat 12 patients with nonhematological malignancies [10]. The drug consists of 1 mg of *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanylglyceryl dipalmitate (DTP-GDP) incorporated into liposomes containing 175 mg of 1-palmitoyl-2-oleoyl-phosphatidylcholine and 75 mg of 1,2-dioleoylphosphatidylglycerol.

Patients enrolled had non-small-cell lung cancer (4), prostate cancer (4), colon cancer (3), melanoma (1) or an unknown primary site of disease (1). One of the patients with colon cancer was treated twice. Three patients per group were each to receive a fixed dose of 200, 400, 600, 800 or 1200 μg/m² weekly, over 4 weeks. Because of acceptable toxicity, the length of treatment was increased to 12 weeks. All patients were hospitalized for treatment and received frequent vital sign monitoring after the injection of liposomal DTP-GDP (L-DTP-GDP).

As expected, fever, chills, and nausea were the most common toxicities; however, asymptomatic hypotension was observed in almost half the patients. In all cases, plasmanate (human plasma protein) infusion without pressors was adequate to correct the blood pressure. All patients were maintained on an infusion of D5 1/2 NS at 100 ml/h for at least 8 h. A decrease in the white count was noted at 90 min, whereas a leukocytosis was noted at 8 h which was due entirely to an increase in neutrophils. A monocytosis or lymphocytosis was not observed. There was no clinical or laboratory evidence of a coagulopathy or of renal, hepatic, or cardiac toxicity. In seven of the eight patients who had pulmonary function tests, a decrease in diffusing capacity of 8.0–17.9% was noted.

Several biologic responses were noted. A lymphocyte nadir occured at 4 h. Interestingly, the monocyte population was significantly higher during the first treatment compared with the subsequent three treatments. A consistent non-dose-dependent decrease in NK activity was noted after all treatments. With the exception of one patient, the nonresponders had higher levels of NK activity than the responders. Monocyte cytotoxicity decreased during the first 24 h, but returned to normal before the next treatment. Unstimulated monocyte activity at 168 h was equivalent to that of DTP-GDP-stimulated activity, suggesting a prolonged monocytic activation. Maximum TNF levels were noted at 1.5–2 h after treatment and returned to pretreatment

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Clinical toxicities in the four liposomal muramyl peptide trials were similar. Chills, fever, nausea, vomiting and malaise were the most common side effects. Interestingly, while both hypertension and hypotension were observed in the L-MTP-PE trials, half of the patients receiving liposomal L-DTP-GDP experienced only hypotension. While malaise was the dose-limiting toxicity noted by Murray, none of the other investigators reported significant dose-related toxicity. The maximum dose administered by Vosika (1.2 mg/m^2) [10] was only a fraction of the 6.0 mg/m$_2$ dose tolerated in the L-MTP-PE trials. It does not appear that biweekly dosing of L-MTP-PE significantly affects toxicity or tumor response.

The rationale for combining chemotherapy with L-MTP-PE has been discussed. The argument against using concurrent chemotherapy with macrophage activators is that the chemotherapeutic agent may inhibit macrophage function. Kleinerman et al. performed monocyte macrophage-mediated cytotoxicity assays on peripheral blood samples from children with osteosarcomas who were treated with chemotherapy [44]. It was determined that single-agent chemotherapy consisting of cisplatin, high-dose methotrexate, cyclophosphamide, or doxorubicin did not interfere with monocyte function. However, when doxorubicin was given on the same day as cyclophosphamide, a marked suppression of monocyte activation was noted. Therefore, it is recommended that L-MTP-PE not be administered with combination chemotherapy containing cyclophosphamide and doxorubicin. Other combinations of chemotherapy require similar in vitro testing before they can be recommended for clinical trials involving monocyte activation.

Pathologic changes observed in five patients who received MTP-PE for pulmonary metastases that occurred during adjuvant chemotherapy or persisted despite chemotherapy for osteosarcoma were reported by Kleinerman [45]. MTP-PE was prepared by CIBA-GEIGY as previously discussed. Eligible patients were rendered disease free by surgery, then given 2.0 mg/m^2 MTP-PE twice monthly for three months. The pathologic changes of tumor metastases from patients who had disease recurrence within 6 weeks after completion of therapy was reported.

Biopsy specimens from three patients demonstrated peripheral fibrosis surrounding the tumor with central inflammatory cell infiltration and neovascularization. One patient showed early fibrotic changes, and the other was noted to have a change in histology from high-grade malignant characteristics before therapy to low grade after therapy. Although the results are preliminary, the observation of responses in the periphery with central neovascularization suggests a theoretical advantage for the combined use of L-MTP-PE with chemotherapy. The clinical results of this trial have not yet been published.

V-B. Intraperitoneal L-MTP-PE activated macrophages

In a related study, Faradji et al. conducted a phase I trial that involved the intraperitoneal administration of activated monocytes to patients with peritoneal carcinomatosis [46]. L-MTP-PE was used to stimulate autologous macrophages ex vivo.

Monocytes were separated by the method of counterflow centrifugal elutration, then incubated for upto 18 h in the presence of 1 μg/ml L-MTP-PE provided by CIBA-GEIGY, Ltd. (Basel, Switzerland). This was the same liposomal preparation used by Murray as discussed previously.

The activated monocytes were infused through a Port-A-Cath access system into the peritoneal cavity over a 5 min period between two flushes of 200 ml of Ringer solution. Treatment was performed weekly for 5 consecutive weeks. Each patient received a dose-escalating schedule of $1 - 10^7$ to $1 - 10^9$ cells per week. Five patients had underlying ovarian carcinoma, two patients had pancreatic cancer, and there was one patient each with malignant mesothelioma, gastric cancer and appendiceal cancer. Although all patients with ovarian cancer received CAP chemotherapy, patients with gastric, appendiceal and pancreatic tumors received no prior chemotherapy.

No severe or life-threatening toxicities occurred. Fever, chills, and abdominal pain were the most common side effects. Grade 2 dyspnea was noted in two patients. Increases in fibrinogen, CRP and neopterin suggested a biologic response. Gamma camera images demonstrated a rapid diffusion of tracer in the abdomen with virtually no differences in biodistribution after 7 days. Less than 0.5% of the ^{111}Indium activity was noted in the peripheral blood. At the highest practical infusion level (10^9 monocytes), no dose-limiting toxicity was observed. The clinical efficacy of this phase II trial was not discussed; however, it was noted that the intraperitoneal administration of activated monocytes is safe in patients with peritoneal carcinomatosis and may have an adjuvant therapeutic role in patients with resected ovarian cancer who have minimal residual disease.

V-C. *Tumor-associated antigens*

Phillips, in 1990, described a clinical trial in which liposomes were used to deliver melanoma-derived tumor-associated antigens (L-TAAs) in an attempt to induce active-specific immunomodulation [47]. Thirteen patients with advanced melanomas were treated. Toxicity was limited to a mild tenderness at the injection site; however, no end-organ or hematologic toxicity was noted. There was no mention of constitutional symptoms. In contrast to trials with L-MTP-PE, there were three complete responses and two mixed responses. Three patients were free of disease at the completion of the trial, with response durations of 26, 102 and 110 weeks.

In vitro assays of L-TAA-induced peripheral blood lymphocyte proliferation and antitumor activity successfully predicted clinical responses. Interestingly, all patients with normal natural killer cell (NK) activity prior to therapy had a reduction in NK activity during treatment. This trial demonstrated that liposomal delivery of TAAs is safe and demonstrated a significant response rate in a tumor that rarely responds to conventional chemotherapy. Since no myelotoxicity was observed, the combined use of L-TAAs and cytotoxic chemotherapy would be a reasonable approach to the treatment of patients with advanced melanoma.

VI. Summary

Technological advances in liposomal preparation and efficient drug entrapment, along with supportive preclinical studies, have led to a number of recent clinical trials utilizing liposomes as drug carriers in the treatment of human malignancy. Although the results of these trials must be considered preliminary, it is clear that liposomal delivery of chemotherapeutic agents is safe at the doses administered. Aside from minor constitutional symptoms, virtually all toxicity could be attributed to release of the incorporated drug. Myelosuppression tends to be the dose-limiting toxicity with free drug, whereas constitutional symptoms are more likely to occur with encapsulated biologic therapy. Prior to human trials, there was fear that intravenous injection of liposomes could result in pulmonary emboli. No cases of pulmonary embolism secondary to liposome therapy have been recorded.

The objective response rate in the patients studied appears to be minimal. This is not surprising, since the overwhelming majority of patients studied had disease that was advanced and previously shown to be refractory to therapy. Subgroups of patients that appear to benefit most include those with breast cancer who were treated with liposomal doxorubicin and those with advanced melanoma treated with liposomal tumor vaccines. Additional phase II and III clinical trials will better define the effectiveness of treatment modalities incorporating liposomes.

VI-A. *Future directions*

One of the earliest applications of liposomes may be in the amelioration of drug toxicity. Although not yet proven, the clinical studies reviewed suggest that liposomal delivery of doxorubicin reduces cardiotoxicity without sacrificing antitumor effect. Although similar claims have been made in support of continuous infusion doxorubicin [11], one can avoid unnecessary hospitalization or the bulk and expense of portable infusion devices by a single administration of the liposomal preparation.

Liposome encapsulation can markedly alter the biodistribution and pharmacokinetics of well-known chemotherapeutic agents. The effectiveness of liposomal drug delivery in human trials thus far has probably been more closely related to altered pharmacokinetics rather than enhanced drug delivery to tumor or increased tumor responsiveness.

As demonstrated by Gabizon [19], increased liposome circulating time in the murine model can be achieved by using small unilamellar vesicles containing a phosphatidylcholine of high phase-transition temperature and a small molar fraction of monosialoganglioside or hydrogenated phosphatidylinositol. More recently, Klibinov [48], Mori [49] and Allen [50] have shown that the addition of a 2000 Da (polyethylene)glycol molecule to the liposome surface markedly prolongs circulation time.

Sophisticated vesicles that can avoid the RES and specifically target tumor via ligands such as antitumor monoclonal antibodies have proven effective in animal models and hold promise for future clinical trials. Watanabe et al. were able to demonstrate an antitumor response in nude Mice inoculated with a human melanoma cell line, using an anti-human melanoma antibody (A375) conjugated to liposomes containing macrophage-activating factor [21]. Hashimoto et al. designed immunoconjugates encapsulating actinomycin D with a surface antibody specific for mouse mammary tumors. These immunoconjugates demonstrated antitumor activity in vivo [22]. Similarly, Watanabe et al. have successfully used sodium butyrate laden liposomes conjugated with anti-CD19 to treat a murine lymphoma model [23].

In addition to altering the biodistribution of drugs, liposomal encapsulation appears to alter mechanisms of drug uptake and metabolism. Lovo cells known to be

resistant to free cisplatin have demonstrated sensitivity to L-NDDP. Thus, liposomal delivery may be effective in overcoming mechanisms of cellular resistance and allow the use of encapsulated antitumor agents after resistance develops to the free drug. By overcoming cellular resistance, tumors inherently refractory to a specific agent may become sensitive. Therefore, liposomal encapsulation may increase the spectrum of well-known agents.

As mentioned earlier, there is a rationale for combining liposomal immunotherapy with biological response modifiers. Immunomodulators, such as γ-interferon or interleukin-2 (IL-2), have the potential to augment the effectiveness of liposomal MTP-PE by increasing the antitumor response directly or by allowing vascular leaks such that vesicles have access to the tumor bed. Lautersztain et al. have treated 33 patients afflicted with a variety of metastatic solid tumors refractory to chemotherapy with a combination of gamma-interferon and L-MTP-PE [45]. They demonstrated a clinical response in two patients. The major side effects noted were chills, fever, anorexia, fatigue and reversible leukopenia. Further trials of liposomes in conjunction with biologic response modifiers are under way.

Liposomal delivery of antitumor therapy is in its infancy and the optimal liposome/drug formulation has not yet been determined. However, the clinical implications of objective responses to LED in patients with advanced breast cancer and to L-TAA in patients with metastatic melanoma are significant. Other preparations, such as L-NDDP and L-MTP-PE, which have been well tolerated in phase I studies, require further clinical evaluation. Based on the results of early clinical trials, phase II and III studies are indicated to determine the role of liposome-encapsulated drugs in the treatment of human malignancy.

Acknowledgement

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EXHIBIT 3

delivery to RBS

Drug Delivery Systems. 1. Site-Specific Drug Delivery Using Liposomes as Carriers

Vasant V. Ranade, PhD

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Drug delivery systems, offering controlled delivery of biologically active agents, are rapidly gaining importance in pharmaceutical research and development. To achieve controlled drug delivery, i.e., the administration of drugs so that optimal amount reaches the target site to cure or control the disease state, increasingly sophisticated systems containing different carriers have been developed. Macromolecules represent one of the carriers involved, and they have taken on a significantly prominent role in various modes of administration of therapeutic agents. Among macromolecules, for example, synthetic copolymers, polysaccharides, liposomes, polyanions and antibodies, as drug carriers, liposomes have proved most effective for diseases affecting the reticuloendothelial system and blood cells in particular.

Liposomes, which are vesicles consisting of one or more concentrically ordered assemblies of phospholipids bilayers, range in size from a nanometer to several micrometers. Phospholipids such as egg phosphatidylcholine, phosphatidylserine, synthetic dipalmitoyl-DL-α-phosphatidylcholine or phosphatidylinositol, have been used in conjunction with cholesterol and positively or negatively charged amphiphiles such as stearylamine or phosphatidic acid. Alteration of surface charge has been shown to enhance drug incorporation and also influence drug release. Because of the multifold characteristics as drug carriers, liposomes have been investigated extensively as carriers of anticancer agents for the past several years. Liposomal entrapments include a variety of pharmacologically active compounds such as antimalarial, antiviral, anti-inflammatory and antifungal agents as well as antibiotics, prostaglandins, steroids and bronchodilators to name a few. The liposomal entrapment has been shown to have considerable effect on the pharmacokinetics and tissue distribution of administered drugs.

Despite the potential value of liposomes as unique carriers, the major obstacles are the first order targeting of a systemically given liposomes, physical stability and manufacture of the liposomal products and these problems still remain to be overcome. Drug delivery systems evolving in the 1980s have become increasingly dependent on fundamental cell-biology and receptor-mediated endocytotic mechanisms. Drug delivery systems during the 1990s may take advantage of the specificity of receptor-mediated uptake mechanisms as well as polymer chemistry and cell-biology in order to introduce more precise and efficient target-specific delivery systems that are based especially on the liposome technology.

Over the past nearly three decades, significant advances have been made in drug delivery technology. This effort that was pioneered by Alza Laboratories of Palo Alto, California,[1,2] has been accelerated in recent years because there has been a substantial decline in the number of new drugs available to the medical profession. Pharmaceutical research in the area of drug discovery, especially in the molecular manipulation, that could result in the subsequent development of new chemical entities has not been productive enough. Drug delivery system has thus offered help in efficiently optimizing drugs, in some instances. Drug delivery has now become a multidisciplinary science consisting of recently conceived and rapidly emerging disciplines such as biopharmaceutics and pharmacokinetics, among others. Great strides have been made to achieve success by physical biochemists, pharmacists and other pharmaceutical research scientists working in the university and industrial laboratories.[3–6]

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The underlying principle that the drug delivery technology can bring both therapeutic and commercial value to the health care products has been widely accepted. Recently, large innovator/pioneer companies started losing their market share to the generic competitors with increasing rapidity after their patents expired. This created an intense need for presenting old drugs in new forms that is, in new delivery systems. The pharmaceutical and drug delivery companies that prudently initiated, developed and manufactured these drug-specific systems now seem to enjoy a good return on their investment in the form of increased revenues and market shares.[7]

In the United States, the Drug Price Competition and Patent Term Restoration Act (also known as ANDA-exclusivity provisions act) was passed in 1984, and has provided new incentives to manufacturers who can distinguish their products from the competition by features such as longer dosage schedules, better safety profiles, new indications for existing drugs or new combinations, all of which would only be possible with the use of rate-controlled delivery systems.[8]

The review articles that comprise the entire area of research and development of drug delivery have been divided into four sections, 1. site-specific drug delivery; 2. implantable drug delivery; 3. oral drug delivery; and 4. transdermal, intranasal, ocular and miscellaneous drug delivery.

This review describes site-specific drug delivery systems using liposomes as the carriers for various curative agents. Subsequent reviews in the future will cover other aspects of drug delivery systems.

Drug delivery, which now takes into consideration elements such as the carrier, the route and the target has evolved into a strategy using processes or devices designed to enhance the efficacy of therapeutic agents through a controlled release. This may result in either enhanced bioavailability, enhanced therapeutic index and symptomatic relief, reduced side effects and drug toxicity and/or improved patient acceptance or compliance. Interestingly, controlled drug delivery or release has been defined by Flynn as "the use of whatever means possible, be it chemical, physiochemical or mechanical, to regulate a drug's access rate to the body's central compartment or in some cases directly to the involved tissues." [9]

Although site-specific drug delivery may also imply inherent controlled-release mechanisms, the discussion on this aspect will be dealt with in other parts of this series, e.g., oral drug delivery, where the delayed, prolonged, sustained or controlled release action of a specific drug is more directly and closely related to those delivery systems.

Tomlinson in his comprehensive article[10] on the site-specific drug delivery briefly describes the rationale for this delivery as consisting of features such as: exclusive delivery to specific components, access to primarily inaccessible sites, protection of drugs and body from unwanted deposition, controlled rate and modality of delivery to pharmacological receptor and reduction in the amount of active principle employed. He also described the properties that appear to be needed for site-specific carriers: properties that are biological, drug-related and carrier-related. Tomlinson also lists types of particulate carriers and the material used. These contain carriers such as lipid, polymeric, proteinaceous, carbohydrate and natural. The types of release from particle carriers and the factors affecting these releases are also described.[10,11]

LIPOSOMES IN DRUG DELIVERY

Most efforts to make drug therapy more efficient by direct delivery of drugs to affected tissues have focused on "local" or "regional" injection such as intra-arterial infusions and infusions into body cavities such as the peritoneum. The benefits of regional therapy have been a reduction in systemic toxicity and peak drug levels directly at the site of affected tissues. However, these methods of administration have met with limited success. For example, although intra-arterial injections effectively concentrate drugs at tumor sites in some cases, in others, they clear the drug from the system so rapidly that the benefits are not realized.

Currently, the pharmaceutical researchers are trying to design drug delivery systems so that the drugs will localize and affect only the afflicted tissues. The most promising carrier system for drugs at this time is liposomes.[12-17]

Liposomal affinity for various tissues can be changed by making liposomes containing phospholipids with various fatty acid chain configurations so that these microparticles may be either solid or liquid at defined temperatures.[18,19] Altering the charge on the liposomes can greatly influence their distribution in the body. Negatively charged vesicles enter the cell by fusion, which allows the drug to be discharged into the cell cytoplasm. Neutral vesicles are incorporated into the cell by phagocytosis, which exposes the drugs to the lysosomal digestive system of the cells. Positive and neutral liposomes are cleared more slowly than negative ones. These factors allow researchers to design specific liposome carriers for certain pharmacologic agents.

What is the liposome made of and how does it look? The liposome is a microparticulate ranging in size from 0.03 μm to 10 μm and consists of a bilayer

of phospholipid encapsulating an aqueous space. A variety of amphipathic lipid molecules can be used to form the bilayer.[20] The size and morphology of the structure are regulated by the method of preparation. The lipid molecules arrange themselves by exposing their polar head groups towards the water phase, while the apolar hydrocarbon moieties stick together in the bilayer, thus forming closed concentric bimolecular lipid leaflets separated by aqueous compartments.

Drug molecules can either be encapsulated in the aqueous space or intercalated into the bilayer, (Figures 1 and 2) the exact location of the drug in the liposome will depend upon the physiochemical characteristics of the drug and the composition of the lipids. Formation of stable liposomes from phospholipids is only possible at temperatures above the 'gel to liquid-crystalline' phase transition temperature (Tc) which represents the melting point of the acyl chains. All phospholipids have a characteristic Tc,[21] which depends on the nature of the polar head group and on the length and degree of unsaturation of the acyl chains.[22] Above Tc, phospholipids are in the liquid-crystalline phase, characterized by an increased mobility of the acyl chains. Decrease in temperature below Tc induces a transition to a more rigid state ('gel state') resulting in restrained mobility of tightly packed acyl chains. When the lipid molecules arrange themselves to form closed bilayer structures, water and solutes, (e.g., drugs), are trapped between adjacent planes of polar head groups.

Liposomes can be formed from a variety of phospholipids. The lipid most widely used is phosphatidylcholine,[23,24] either as such or in combination with cholesterol. Cholesterol is known to condense the packing of the phospholipids in bilayers above Tc, thereby reducing their permeability to encapsulate compounds. To confer surface charge to the liposomes, negatively charged lipids such as stearylamine are used. For drug molecules encapsulated in the aqueous space, the bilayer serves as a diffusion barrier permitting the liposomes to serve as rate-controlling input devices. Papahadjopoulos and coworkers have done pioneering research in trying to establish and develop the liposomal delivery system from experimental therapeutics to clinical applications.[25-29] The introduction of this delivery system directly to the target site (such as the eye, lung or bladder) is a well-established approach for treating local diseases and liposomes have been shown to play a beneficial role in this application.

THE LIPOSOME-DRUG CONCEPT

Liposomes have been used in a variety of routes of administration including intravenous, intramuscular, intraperitoneal and oral.[30-32] However, IV injection is the most widely practical route. The half-lives of liposomes in the blood stream can range from a few minutes to many hours depending on the size and lipid composition of the vesicles. Large size vesicles such as multilamellar or large unilamellar ones are rapidly cleared from the blood and taken up by the cells of the reticuloendothelial (RES) system[33] especially by the macrophages in liver (Kupffer cells) and spleen.[34] Following IV administration, small liposomes (range, 0.1 to 1.0 μm) are taken up preferentially by cells of the RES system located principally in the liver and spleen, whereas liposomes larger than 3.0 μm lodge in the lung. This preferential uptake of intermediate size liposomes by cells of the

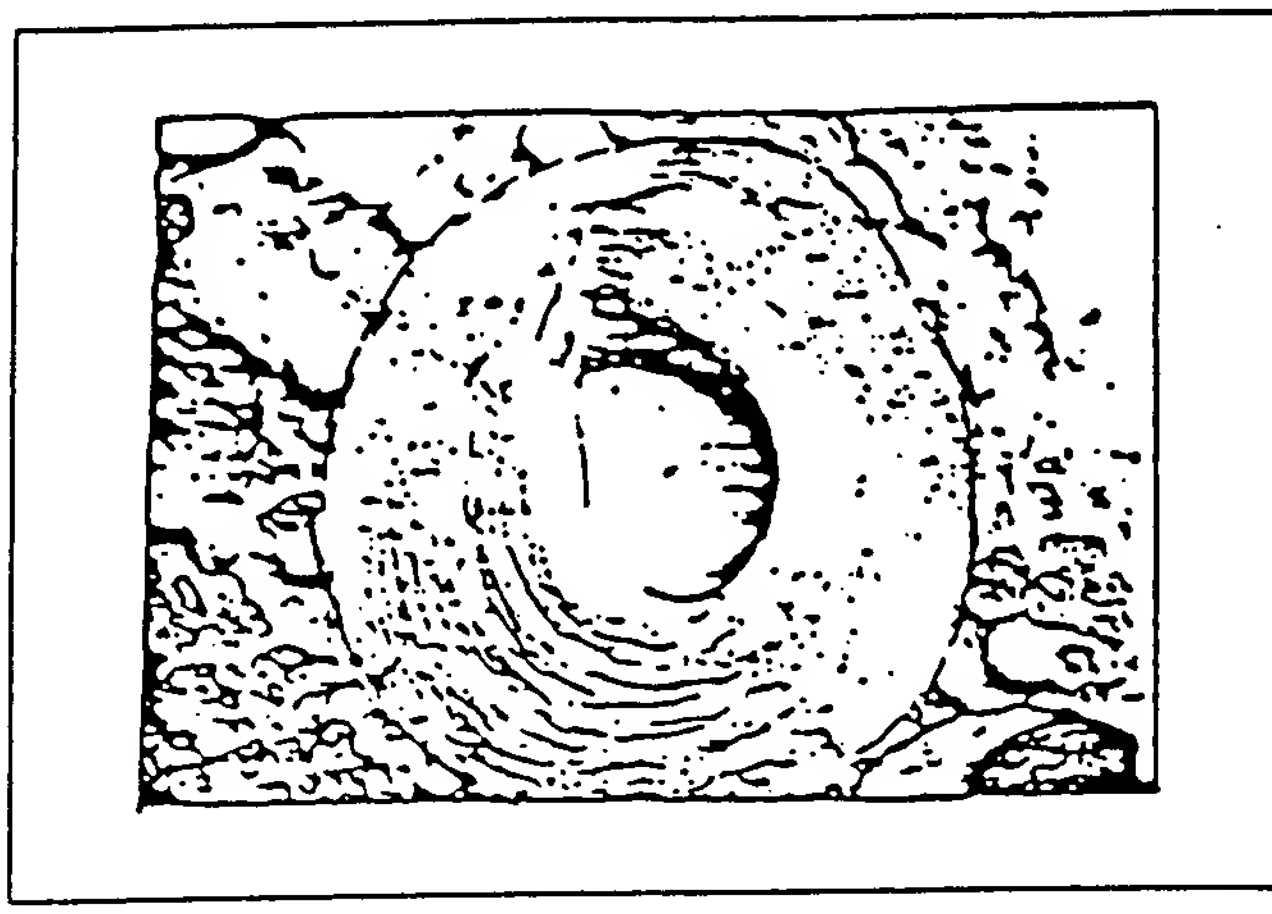

Figure 1. Schematic of a bilayer vesicle or liposome. (Reprinted by permission from Pharmaceutical Technology.) Drug Delivery Systems, Conference Proceedings, October 1985, p 16.

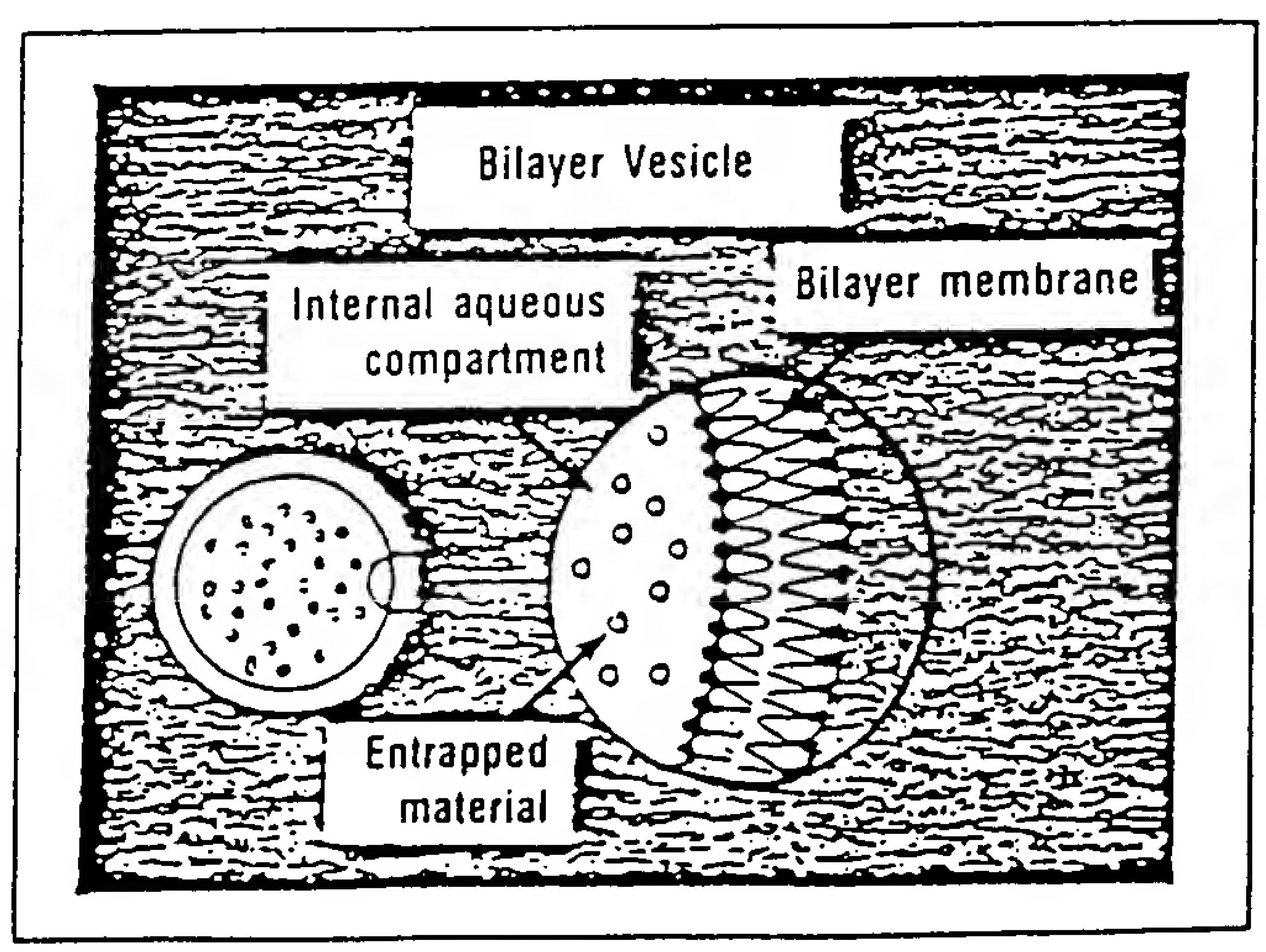

Figure 2. A micrograph view of a liposome. (Reprinted by permission from The Liposome Company Inc.)

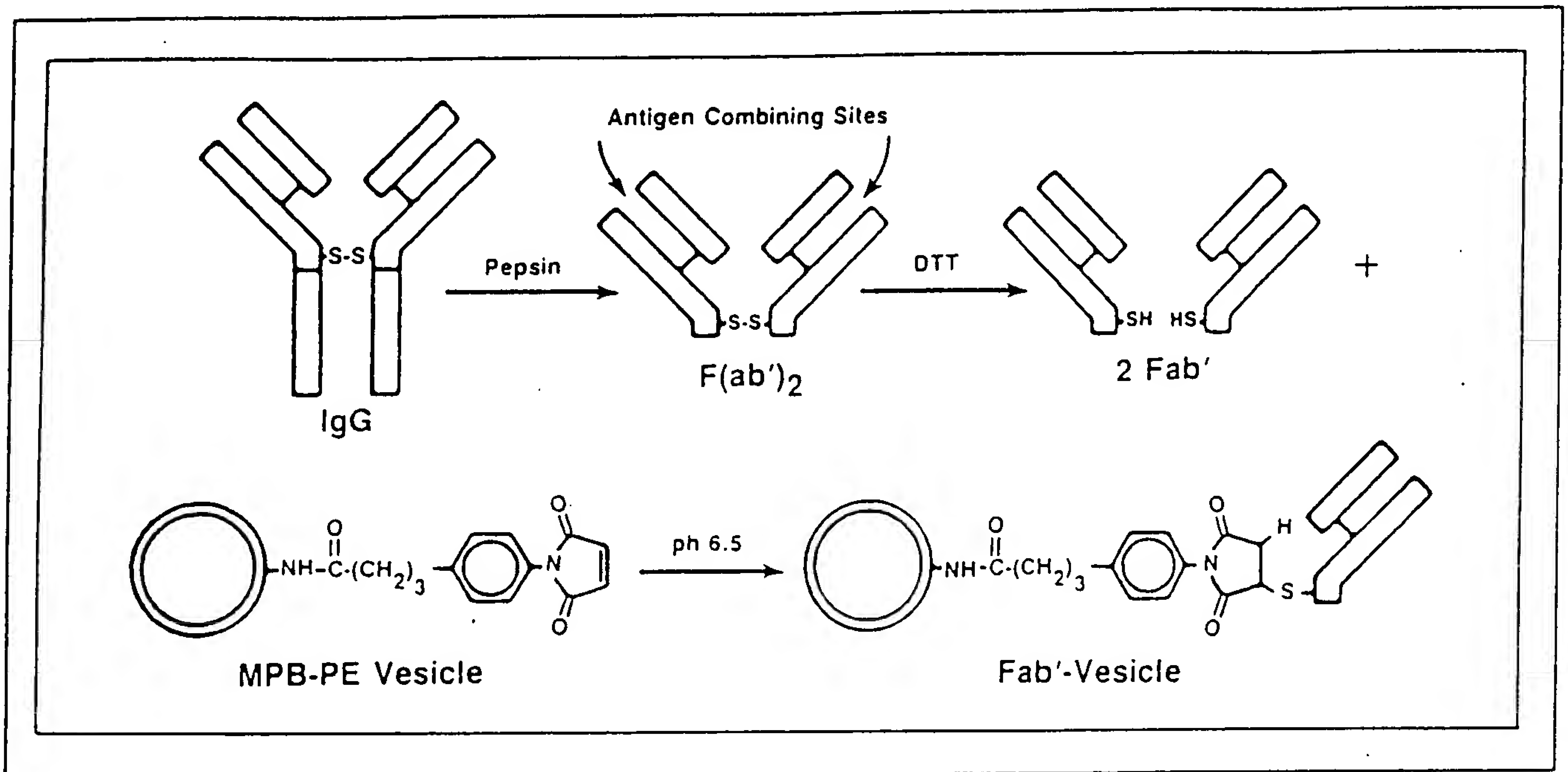

Figure 3. Illustration of the chemical-coupling methodology for antibody/liposomes. (Reprinted by permission from Pharmaceutical Technology.) Drug Delivery Systems, Conference Proceedings, October 1985, p 19.

RES system has been exploited to deliver chemotherapeutic agents to macrophages and to tumors of liver.

Alternative physical approaches based upon the ability to destabilize the liposome bilayer have led to the design of heat-sensitive liposomes, light-sensitive liposomes and pH-sensitive liposomes.[35,36] With this approach, the target must either be identified so that perturbation can be applied directly to the site, or the target site must have the requisite physical differences from other sites in the body.[37]

The chemical approach to achieving site-specific delivery requires that the liposome has a ligand attached to its surface to permit it to preferentially attach to the target site. A variety of targeting ligands has been proposed for this purpose, including antitumor monoclonal antibodies, carbohydrates, vitamines and transport proteins.[38] Only the carbohydrate and monoclonal antibody modified liposomes have thus far shown promise of the desired targeting.

Successful targeting of liposomes to cells other than those belonging to the RES, with the exception of the hepatocytes and possibly, circulating blood cells, is very unlikely.[39] Although a high degree of specific liposome-cell association was obtained in vitro by coating the vesicles with cell specific ligands such as monoclonal antibodies or $F(ab')_2$ fragments,[40–42] an efficient transfer of encapsulated drug was not always observed (Figure 3) In vivo, the obstacles which have to be overcome are formidable. Firstly, the liposomes have to escape from nonspecific clearance by the RES cells. Secondly, the vesicles have to cross the capillary endothelium and the basement membrane, and thirdly, many cell types, including most tumor cells, display only a very low endocytotic capacity. Since endocytosis is the dominant mechanism of liposome-cell interaction, this means a serious limitation to the successful application of liposomes as a drug delivery system.

Liposomes appear to be an ideal system for the selective delivery of drugs to cells belonging to the RES. Small-size liposomes may also serve as carriers of drugs to be delivered to liver parenchymal cells by virtue of their capacity to penetrate the fenestrated liver endothelium. Once taken up by the cells the liposomes are degraded in the lysosomal compartment. Liposome-encapsulated drugs, when resistant to the intralysosomal environment, may slowly leak out of the lysosomes into the cytosol and could become available to exert their therapeutic action. The drug may also be released by the macrophages so as to maintain a therapeutic drug level in blood and/or tissues for a prolonged period of time. The sustained release of drugs which may occur from circulating liposomes, liposomes adsorbed to cell membranes as well as from macrophages which have engulfed drug-loaded liposomes, may be an

important application of liposomal delivery systems. Another interesting aspect of the liposome-drug concept is the reduction of the toxicity of liposome-encapsulated agents. This might be particularly true for antineoplastic agents such as Adriamycin or antimicrobial drugs like Amphotericin B.[43–45] The decrease in toxicity is thought to be accomplished by altered pharmacokinetics and tissue distribution of the drugs applied.

LIPOSOMES AS CARRIERS OF THERAPEUTIC AGENTS

Since 1972 when Gregoriadis proposed the use of liposomes as carriers of enzymes in the treatment of lysosomal storage diseases, the application of liposomes has been extended to a variety of drugs such as antineoplastic agents,[15,46,47] antimicrobial compounds[42,48] and immunomodulators.[49–52] Besides application of liposomes as drug carriers in the treatment of intracellular infections,[53] these vesicles have also been used as carriers of Amphotericin B in the treatment of mycotic infections such as histoplasmosis cryptococcosis[54] and candidiasis. In a preliminary clinical study Lopez-Berestein and colleagues found that liposomal Amphotericin B was effective in the treatment of Candida and Aspergillus infections in leukemia patients, who had previously failed to respond to treatment with the non-encapsulated drug.[55] The increase in therapeutic efficacy of amphotericin by encapsulation in liposomes apparently results from a reduction of toxicity of the drug. In a recent study Juliano and coworkers[56] found that, while free amphotericin is extremely toxic to both fungal cells and mammalian cells in vitro, the liposomal drug remains toxic to fungal cells but has little effect on mammalian cells. It is well known that amphotericin interacts with ergosterol in fungal cell membranes forming transmembrane channels resulting in extracellular release of ions and metabolites. On the other hand, the drug also interacts with cholesterol in mammalian cell membranes, which is probably the basis of its toxicity. Incorporation of lipophilic amphotericin within liposomes might result in a facilitated transfer of the drug to fungal cells, while transfer to mammalian cells is hampered. This selective transfer of amphotericin from liposomes to fungal cells may form the molecular basis of the reduced toxicity in addition to other factors such as altered kinetics or tissue distribution.[57]

Another interesting example of enhancement of antibacterial activity by liposome encapsulation was published by Sunamoto and coworkers.[58] They showed that uptake of IV injected liposomes by circulating monocytes and alveolar macrophages can be increased by coating the vesicles with a palmitoyl derivative of amylopectin. After IV injection, the amylopectin-modified liposomes were found to distribute to the lungs with high preference.

The natural avidity of macrophages for liposomes can also be exploited in the application of the vesicles as carriers of immuno-modulators to render these cells cytotoxic to metastatic tumor cells. Macrophages appear to be an important barrier against the proliferation and metastatic spread of tumor cells. Activation of macrophages to tumor cytotoxicity occurs as a result of exposure to a variety of immunomodulating substances such as lymphokines,[59] γ-interferon and muramyl dipeptide (MDP).[60–63] Liposomes are believed to increase adjuvant activity of MDPs. Adjuvants are nonspecific immune stimulants that boost immune responses to weakly antigenic molecules. MDP micelles are very potent adjuvants in tests for vaccination against bovine viral diarrhea. By an unknown mechanism, activated macrophages are capable of selectively killing tumor cells, thereby leaving normal cells unharmed. This approach may open new perspectives in the management of metastatic cancer which is often seriously hampered by the biological heterogeneity of the tumor cells with respect to growth rate, sensitivity to various cytotoxic drugs etc.

Although preliminary results with liposome-encapsulated immunomodulators are encouraging, successful application of these in the treatment of patients with liver metastasis may be hampered by unfavorable macrophage:tumor cell ratios in many metastatic tumors.[64] Therefore, therapeutic regimens designed to stimulate microphage-mediated tumor cytotoxicity almost certainly will have to be used in combination with other treatment modalities such as chemotherapy to reduce the tumor load, while activited macrophages could eradicate surviving tumor cells.[65–67]

Successful targeting of liposomes, at least to solid tumors located outside the bloodstream, is very unlikely to be achieved. Selective introduction of antineoplastic drugs into tumor cells in vivo by means of liposomes would seem to be an impossible task at the present time. On the other hand, application of liposomes as a drug delivery system for antitumor drugs may be of great benefit in diminishing toxicity of encapsulated compounds by altering the pharmacokinetics and/or tissue distribution. In addition, liposomes can serve as a sustained- or controlled-release system for cytostatic drugs such as cytosine-arabinoside. The therapeutic effect of this cell-cycle-specific drug is enhanced by liposomal encapsulation possibly by maintaining therapeuti-

does amylopectin cause lung uptake?

cally favorable drug levels for a prolonged period of time following leakage from the liposomes or, alternatively, from macrophages that have phagocytosed drug-loaded liposomes.

A promising example of a liposomal delivery system for antitumor drug is the use of doxorubicin (DXR) in liposome-encapsulated form. Doxorubicin, an anthracycline antibiotic, has a well-established position in the treatment of a variety of solid neoplasms, lymphomas and leukemias. Its clinical use, however, is limited by its cardiotoxicity. Several investigators have shown that entrapment of DXR within liposomes markedly reduces its cardiotoxicity without loss of antitumor activity.[68–71] However, the mechanism responsible for the increased therapeutic index is not fully understood. Low uptake of the liposomal DXR by the myocardium in addition to depot formation in macrophages leading to sustained release of the drug into the circulation or directly to target tumor cells might be held responsible for the reduction in (cardio)toxicity and preservation of antitumor activity.

Uptake and processing of DXR-liposomes by mononuclear phagocytes of the RES seem to be important determinants of the in vivo therapeutic action of the drug. The beneficial effect of liposomal encapsulation (reduction of cardiotoxicity along with preservation of antitumor activity) is thought to be the result of prolonged DXR levels maintained by infusion of DXR (or active metabolites) into the blood from these depot cells. By manipulating the lipid composition of the liposomes, rates of intracellular degradation can be influenced, which, in turn, will determine the rate of release of the drug and thereby, conceivably, its therapeutic effect.[34,72] Crucial for such a drug delivery system is of course that DXR is released in a cytotoxic form from the macrophages. Recent observations with peritoneal macrophages revealed that this is a real possibility.[72] Penetration of intravenously injected liposomes to other tissues such as the myocardium, skeletal muscles or the central nervous system is very poor because of the tight endothelium lining of the capillaries in these organs preventing egress of the vesicles from the vascular compartment.[64]

many release of drug after uptake

During their stay in the bloodstream, liposomes may be susceptible to destabilizing effects of serum proteins, resulting in leakage of encapsulated water-soluble compounds. High density lipoproteins (HDL) have been found to be mainly responsible for penetration of liposomal bilayers, a process which was accompanied by net loss of phosphatidylcholine from the liposomes to the HDL.[73,74] Very high susceptibility was found at the gel-to-liquid phase-transition temperature of the liposomal lipid, while both above and below that temperature, the liposomes were relatively insensitive.[75] However, net loss of phospholipid could be prevented and concomitantly, retention of encapsulated drugs improved by incorporation of cholesterol in the liposomal membranes, thereby impending penetration of serum lipoproteins, and thus resulting in an increased stability of liposomes.

Mufson has recently discussed liposome formulations for aerosol administration of a histamine bronchodilator as a form of delivery system to the lung.[76] Other significant antibody-targeted liposomes contain actinomycin D and successful entrapment of diagnostic reagents such as LEAP. In both classes of targeting, namely, site-specific type and site-avoidance type, the liposomes have demonstrated an ability to improve the therapeutic index of currently used drugs. It appears reasonably likely that these biocompatible, biodegradable capsules that migrate to sites of infection, tumor or inflammation, will command greater attention of pharmaceutical industry as it seeks to increase the therapeutic index of its drugs while simultaneously gaining product exclusivity via proprietary liposome technology.

Recent studies and examples of the liposomal formulations containing the various entrapped active ingredients follow.

1. A liposome product to deliver medication to the eye (for example, dry eye syndrome and glaucoma). An ocular lubricant consisting of water contained in bioadhesive liposome could be used.
2. Liposomes can be used for oral delivery if the target is the stomach or the intestine.[77]
3. Animal studies have been carried out using liposomes and heat to deliver anti-cancer drugs. Cancer-drug containing liposomes are injected into the blood stream. But, at temperatures a few degrees above normal, the liposomes melt allowing drugs to leak out.
4. Liposomes may be able to be used to target cancer-killing biologicals, such as interleukin, to overproliferating white blood cells.
5. Primaquin (an antimalarial agent) has been coupled to a liver cell targeting peptide to form a complex which can be encapsulated
6. A new liposomal product known as proliposome was developed which has a significantly stronger membrane.[78,79]
7. Multivesicular liposomes for the administration of anticancer agents. These liposomes are composed of multiple, non-concentric aqueous chambers, allowing more efficient drug entrapment and improved incorporation of drugs, including cytarabine and bleomycin.

8. 2,3-Dideoxycytidine (DDC)-anti-HIV antiviral. Liposomes were manufactured as a complex of dioleoyl lecithin, dipalmitoyl phoaphatidyl glycerol, cholesterol and triolein which encapsulate the active ingredient.
9. The antitumor activity of macrophages can be directed against metastases. If a primary tumor is removed, Macrophage Activation Factors (MAF) found in the body can stimulate macrophages of the body's immune system to attack the secondary tumors. Liposome entrapped MAF were found to be free of metastases.
10. L-NDDP (cis-bis-neodecanoato-trans-R,4-1,2-diaminocyclohexane platinum)
11. Vincristine
12. Cytochalasin B
13. Vaginal antifungal agent such as miconazole
14. Aminoglycoside antibiotic, gentamicin for Gram negative infections
15. Amikacin as liposome-encapsulated injectable antibiotic
16. A prostaglandin
17. Steroidal liposomes from sterols such as cholesterol, vitamin-D and steroid hormones
18. Benzylpenicillin
19. Topical anti-inflammatory, beclomethasone dipropionate; bronchodilators such as metaproterenol (Metasome) and terbutaline
20. Liposomal non-steroidal anti-inflammatory formulation of indomethacin
21. Daunorubicin (Cerubidine)
22. Radiopharmaceutical VS-101 (Vestar)
23. Cisplatin (Platinol)
24. Minoxidil
25. Calcitonin
26. Influenza and melanoma vaccines using liposomes to enhance immune response
27. Animal antibiotic
28. Bovine somatotropin
29. Immunoliposomes
30. Antimonial drug meglumine antimoniate for Leishmaniasis[80-82]

CONCLUSION

Although liposomes possess a number of favorable properties which would enable them to function as a drug delivery system, there are also severe limitations, the major one is the inability of the vesicles to cross the capillary endothelial cells in most organs except the liver.[83] Another obstacle is the limited potency of many cell types to phagocytose particles like liposomes while, by the same token, fusion with the plasma membrane has proven to be of little if any significance thus far for any cell type.

On the other hand, liposomes seem to be attractive carriers of drugs to macrophages, which is demonstrated by the application of liposomes in the treatment of certain infectious diseases or in the immunotherapy of cancer. Besides that, the use of liposomes appears to be of great benefit in the reduction of toxicity of certain drugs such as amphotericin or adriamycin. In addition, interesting results have been obtained on the application of liposomes for the local delivery of drugs,[84,85] e.g., the intra-articular injection of liposome-associated cortisol palmitate in the treatment of arthritis,[86,87] the intrapulmonary application in the therapy of the respiratory distress syndrome or the intralymphatic administration for the diagnosis or treatment of lymph node metastasis.[88,89] Finally, problems concerning large-scale production of liposomes as a pharmaceutical product, acute and chronic toxicity and immunogenicity of liposome preparations require rigorous attention before clinical application of the vesicles as a drug delivery system can be considered. Klimchak and Lenk have addressed this question very well.[90]

Interestingly, Roerdink in his recent review article[14] has pointed out that however, inspite of some early remarkable successes, liposomes are by no means a panacea for pharmacotherapy in general. His statement still seems to be aptly justified.

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EXHIBIT 4

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Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor

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Summary

Tumor drug uptake and antitumor efficacy of free and liposomal doxorubicin (DOX) were determined in the SC115 Shionogi mouse mammary tumor. Liposomal DOX systems were prepared by pH gradient-driven drug encapsulation in 170 nm egg phosphatidylcholine/cholesterol (55:45, mol ratio) vesicles. Intravenous injection of free DOX at 6.5 mg/kg, the maximum tolerated dose for free drug in the multiple dose therapy regimen, resulted in tumor-associated drug levels of 2.0 μg/g tissue at 1 h which remained constant over 24 h. Liposomal DOX injected at 6.5 mg/kg led to an accumulation of drug in the tumor from 2.6 μg/g tissue to 5.5 μg/g tissue between 1 h and 24 h, respectively. Increasing the dose of liposomal DOX to 13.0 mg/kg increased tumor drug uptake levels to 5.7 μg/g and 10.2 μg/g tissue at 1 h and 24 h, respectively. Administration of free or liposome encapsulated DOX every 7 days for 3 weeks resulted in a dose-dependent decrease in tumor growth rate. However, liposomal DOX injected at 6.5 mg/kg exhibited enhanced tumor growth inhibition compared to an equivalent dose of free drug. Further, the ability to administer increased doses of the less toxic liposomal DOX not only resulted in a greater inhibition of tumor growth but also significantly reduced tumor weight. Tumors weighing as much as 5 g were diminished to less than 0.5 g upon treatment with liposomal DOX at a dose of 13 mg/kg. In addition, groups receiving the highest liposomal DOX dose exhibited 25% complete tumor regression which persisted over the 50-day study period. These results demonstrate the ability of appropriately designed liposomal DOX systems to significantly enhance the delivery and retention of drug at solid tumor sites, resulting in increased therapeutic activity.

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Abbreviations: EPC, egg phosphatidylcholine; Chol, cholesterol; DOX, doxorubicin; MTD, maximum tolerated dose; EDTA, ethylenediaminetetraacetic acid.

Keywords: liposomal doxorubicin; tumor drug uptake; antitumor efficacy.

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Introduction

Numerous studies have addressed the use of liposome encapsulated DOX in the treatment of various tumor types. Results from several laboratories indicate that liposome encapsulation of DOX does not compromise drug potency while buffering toxic side effects [1,6,13,14,18,19,22], most notably cardiotoxicity [1,5,6,13,18,22]. This ability of liposomes to enhance the therapeutic potential of DOX has been demonstrated in a variety of metastatic [6—8,15], ascitic leukemia [13,14,18—20] and solid tumor models [5,19,22].

Although the above studies clearly establish the potential utility of liposome encapsulation in improving the therapeutic activity of DOX, little is known about the mechanism whereby this effect is achieved. For example, recent reports have demonstrated that manipulation of liposome characteristics can result in enhanced vesicle accumulation in solid tumors [9]. However, it is unclear whether this capability to improve delivery of entrapped drugs to tumor sites necessarily increases antitumor efficacy. Investigations by Gabizon et al. [7] employing a metastatic liver tumor model implied that such a relationship may be important in determining antitumor activity. The relevance of this implication to tumors residing at sites that do not inherently accumulate liposomes is still unresolved.

In order to better understand the comparative antitumor activities of free and liposome encapsulated DOX, we have investigated tumor drug uptake characteristics and efficacy behaviour of these systems in the transplantable androgen-responsive Shionogi mouse mammary carcinoma (SC115). This tumor arose spontaneously in a female mouse of the DD/S strain. After 19 passages in male mice, an androgen-responsive variant was isolated [16]. Although this tumor grows in males, selection of this tumor model was based on the observations that this mouse mammary tumor is similar to many human breast cancers in its sensitivity to different classes of steroid hormones, including androgens [2,10], estrogens [17] and glucocorticoids [23]. In addition, we have previously shown that free DOX administered for three weeks at 7 day intervals is effective in causing growth delay of the SC115 tumor [4]. It is demonstrated here that appropriately designed liposomal DOX systems permit the use of higher DOX doses and increases delivery of drug to the tumor, resulting in improved antitumour activity over free DOX.

Materials and methods

DOX was obtained from Adria Laboratories (Mississauga, Ont.). Egg PC was obtained from Avanti Polar Lipids (Birmingham, Al). Cholesterol salts were obtained from Sigma Chemical Co. (St. Louis, MO).

Liposomes were prepared by hydrating a thin film of EPC/chol (55:45 mol ratio) in 300 mM citric acid buffer (pH 4.0) at a concentration of 100 mg lipid/ml buffer with vortex mixing. After freezing and thawing the multilamellar vesicles 5 times, the liposomes were extruded 10 times through 2 stacked 0.2 μm pore size polycarbonate filters [12]. The resulting vesicles had a mean diameter of 170 nm as determined by quasielastic light scattering. The vesicles were then titrated to pH 7.8 with 0.5 M Na_2CO_3. These vesicles were then heated at 60°C for 5 min and added to solid DOX (Adriamycin® from Adria Labs) to achieve a drug-to-lipid ratio of 0.27:1 (w/w). The mixture was subsequently heated at 60°C for 10 min. Under these conditions, greater than 98% of the drug was sequestered into the liposomes as determined by column chromatography [11]. Liposomal DOX solutions were diluted with sterile saline to achieve drug concentrations appropriate for administering proper drug doses to mice in 0.2 ml.

The Class I SC115 subline was used in these experiments [3]. It was maintained by serial transplantation in male mice of the DD/S strain as previously described [4]. Tumors weighing approximately 2 g were dissociated in 0.05% trypsin (1:250) and 0.025% EDTA

(Sigma Chemical Co., St. Louis, MO) in Ca^{2+}- and Mg^{2+}-free saline A (pH 7.3) and the cell suspension centrifuged at 80 × g for 4 min to enrich the epithelial cell population. The pellet was resuspended in Dulbecco's modified Eagle's medium (DME; Terry Fox Laboratory, Vancouver, B.C.) and passed through a 150 μm Nitex filter (Tetko, Inc., Elmsford, NY) to collect single cells and small cell aggregates. Viable cells, determined by Trypan blue exclusion, were counted on a hemacytometer. Suspensions of 3×10^6 cells in 0.1 ml of DME were injected s.c. into the interscapular region of male mice 2—4 months old. Mice were randomly distributed to the different treatment groups ($n = 9$) immediately following tumor cell injection.

Tumor uptake of DOX was determined for free and liposomal DOX at the maximum tolerated dose (MTD) of free drug (6.5 mg/kg, 3 × weekly regimen) as well as for the MTD of liposomal drug (13.0 mg/kg, 3 × weekly regimen). Mice bearing the SC115 subcutaneous tumor (0.5—1.0 g) were injected i.v. with the indicated dose of free DOX or liposomal DOX containing [^{3}H]cholesterol hexadecylether as a lipid marker (200 dpm/μg lipid). At the indicated times mice, (4 per group) were anesthetized with ether and plasma was recovered from blood collected via heart puncture into 'Microtainer' tubes (Becton-Dickenson, Richmond, B.C.) containing EDTA beads. Tumors were removed and stored frozen at −70°C. Tumor liposomal lipid levels were determined by preparing a 10% tissue homogenate and determining the radioactivty in an aliquot of solubilized (employing Protosol, NEN Nuclear, Mississauga, Ont.) tumor by scintillation counting. Plasma and tumor DOX levels were determined by monitoring the fluorescence of extracted samples [2] at 550 nm. Tumor drug and liposomal lipid levels were corrected for endogenous plasma volume contributions in individual mice on the basis of ^{14}C-containing 100 nm distearoylphosphatidylcholine/cholesterol liposome levels in the tumor 5 min after their injection i.v. at a dose of 100 mg lipid/kg. Under the conditions employed, >95% of these liposomes were recovered in the plasma and tissue blood volume corrections can consequently be made for each tumor drug level determination. The validity of such corrections were confirmed by determining tumor blood volumes for representative mice injected with ^{51}Cr-labelled red blood cells.

For efficacy experiments, tumor-bearing mice were palpated 3 times/week until tumors were measurable, after which caliper measurements were made. Tumor weights were calculated according to the formula [21]:

$$\frac{\text{length (cm)} \times [\text{width (cm)}]^2 = \text{g}}{2}$$

Comparisons of calculated and actual (measured) tumor weights indicated that calculated tumor weights were accurate within ± 10%. Upon growth of the tumor to 0.3—2.5 g mice were administered saline, empty liposomes (administered at a dose equivalent to that given for a liposomal DOX dose of 13 mg/kg), free DOX and liposomal DOX i.v. at the indicated doses at 7 day intervals (3 injections of the indicated dose). Mice whose tumors did not reach a size of 0.5 g by day 25 post tumor transplant were characterized as 'no takes' and were removed from the study. Treatment doses were based on the initial animal weights prior to tumor inoculation. Tumor growth was monitored 50 days post first treatment. Statistical significance of differences in the group means of tumor weights were determined employing Student's t-test (2 sided).

Results

The liposomal DOX preparation employed here demonstrated reduced toxicity compared to the free drug. The MTD for free and liposomal DOX was 6.5 mg/kg per injection and 13.0 mg/kg per injection, respectively, for the day 1, 8, 15 i.v. dose regimen utilized in therapy evaluations. At the 13.0 mg/kg liposomal DOX dose, no deaths could be directly related to drug toxicity. In contrast, administration of

free DOX at a dose of 6.5 mg/kg resulted in one toxic related death and a dose of 13.0 mg/kg per injection caused a 70% mortality rate (data not shown). This 2-fold increase in MTD compares well with our previous studies employing similar liposomal DOX systems in other mouse strains [13]. Plasma and tumor drug uptake levels were therefore determined over 24 h post injection for free DOX at 6.5 mg/kg and liposomal DOX at 6.5 mg/kg and 13.0 mg/kg (Table 1). Low plasma DOX levels were observed between 1 h and 24 h after administration of free DOX. The 1-h value of 0.8 μg/ml corresponded to 0.5% of the injected dose being present in the plasma compartment. This value decreased to 0.03 μg/ml plasma at 24 h. Free DOX accumulation in tumor tissue occurred within the first hour after drug injection, yielding approximately 2 μg/g tissue which was maintained over the 24 h time course.

Liposomal DOX administered at a dose equal to free DOX displayed significantly different pharmacological properties. Plasma drug levels at 1 h and 4 h were 20—25-fold higher than observed for free DOX and 2.3-fold higher at 24 h. More importantly, whereas free DOX tumor levels remained constant over 24 h, tumor drug levels increased from 2.6 μg/g to 5.5 μg/g between 1 h and 24 h for liposome encapsulated DOX. It should be noted that the drug to liposomal lipid ratio present in tumor tissue decreased with increasing time. This is due to the fact that the liposomes employed in this study release entrapped DOX while in the circulation (see plasma drug and lipid levels, Table 1). Hence, liposome accumulation in the tumor at later time points resulted in relatively smaller increases in tumor drug levels. Increasing the dose of liposomal DOX to the MTD (13.0 mg/kg) increased both plasma and tumor DOX levels compared to the 6.5 mg/kg dose. The magnitude of the increase in tumor DOX uptake was comparable to the 2-fold increase in liposomal DOX dose.

Figure 1 shows the rate of tumor growth in individual mice treated with saline, free DOX

Table 1. Plasma and tumor doxorubicin levels after i.v. injection of free and liposomal doxorubicin to Shionogi mice bearing SC115 tumors[a].

Sample	Drug dose (mg/kg)	Time (h)	Plasma		Tumor[b]	
			Dox (μg/ml)	Lipid (μg/ml)	Dox (μg/g tissue)	Lipid (μg/g tissue)
Free Dox	6.5	1	0.8 ± 0.13	—	2.0 ± 0.7	—
		4	0.4 ± 0.06	—	2.1 ± 0.8	—
		24	0.03 ± 0.01	—	1.9 ± 0.8	—
Lipodox	6.5	1	19.8 ± 1.2	290 ± 35	2.6 ± 0.4	4.6 ± 1.3
		4	8.2 ± 1.1	143 ± 27	4.2 ± 0.5	23.3 ± 3.1
		24	0.07 ± 0.01	14.7 ± 1.4	5.5 ± 1.1	47.1 ± 16.3
Lipodox	13.0	1	47.5 ± 7.4	421 ± 31	5.7 ± 1.1	19.3 ± 4.5
		4	37.0 ± 5.9	369 ± 41	12.1 ± 2.7	65.0 ± 12.0
		24	1.4 ± 0.4	44.5 ± 19.2	10.2 ± 3.6	102.6 ± 26.3

[a]Mice (4 per group) were injected with free or liposomal doxorubicin via a lateral tail vein. Drug levels represent the mean and standard deviation.

[b]Tumor drug and liposomal lipid levels were corrected for tumor plasma volume contributions as described in Materials and methods. For liposomal doxorubicin groups, drug and liposomal lipid levels were determined by monitoring fluorescence at 550 nm and radioactivity, respectively as described in Materials and methods.

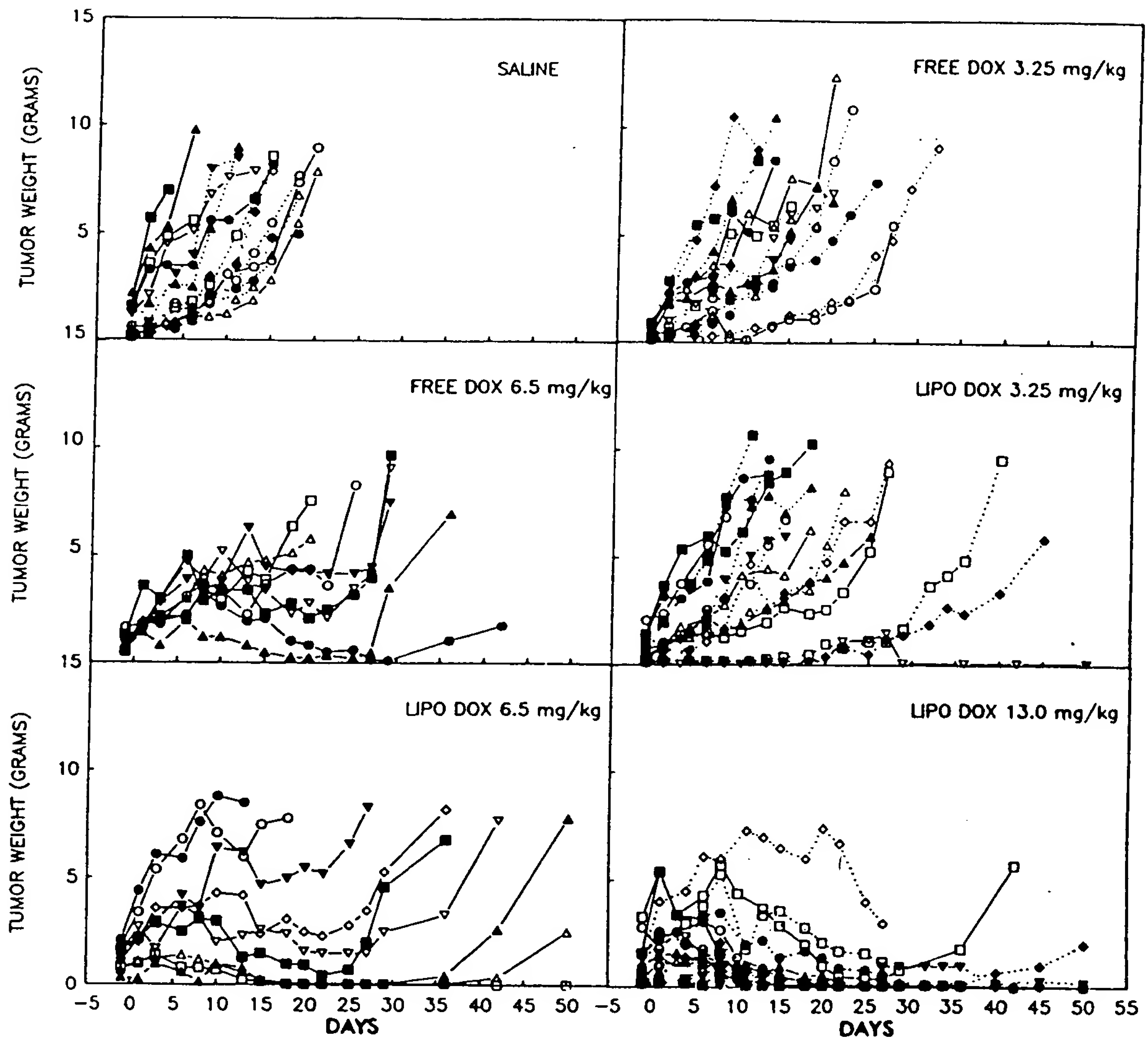

Fig. 1. Tumor growth curves of individual mice receiving the indicated treatments of saline and DOX in free and liposomal form. Experimental conditions are as described in Materials and methods. The indicated drug doses were administered on days 1, 8 and 15. The solid and dotted lines represent separate experiments.

at 3.25 mg/kg per injection and 6.5 mg/kg per injection and liposomal DOX at 3.25 mg/kg per injection, 6.5 mg/kg per injection and 13.0 mg/kg per injection. Mice receiving saline exhibited rapid tumor growth and all animals either died or had to be killed due to debilitating tumor growth within 20 days of the first treatment. Comparable results were obtained for mice administered empty liposomes (prepared identically to drug-containing liposomes) at a dose equivalent to the highest liposomal DOX dose (data not shown). In contrast, administration of DOX in either free or liposomal form caused a dose-dependent decrease in the tumor growth rate.

The relative antitumor activities of the var-

Table 2. Effect of free and liposomal doxorubicin on SC115 tumor growth[a].

Treatment group	Day 1 Tumor wt. (g) Mean ± S.D.	Day 8 Percent increase in mean tumor weight ± S.D. (Compared to day 1)	30-Day survivors[b]	Complete tumor regression[c]
Saline	0.7 ± 0.6	1283 ± 621	0/16	0/16
Free Dox 3.25 mg/kg	0.4 ± 0.3	1178 ± 740	1/14	0/14
Free Dox 6.5 mg/kg	0.9 ± 0.4	307 ± 201[d]	2/8	0/8
Lipodox 3.2 mg/kg	0.6 ± 0.5	743 ± 705	3/16	0/16
Lipodox 6.5 mg/kg	1.3 ± 0.6	135 ± 132[d,e]	6/9	1/9
Lipodox 13.0 mg/kg	0.9 ± 0.8	143 ± 150[d]	13/16[f]	4/16

[a]Mice bearing solid SC115 tumors were treated i.v. with the indicated doses of doxorubicin on days 1, 8 and 15.
[b]Non surviving animals either died or were terminated due to debilitating tumor growth (tumor weight > 7 g).
[c]Complete tumor regression was defined as a non-palpable tumor.
[d]Statistically different from saline control group ($P < 0.01$).
[e]Statistically different from free drug group at the equivalent dose ($P < 0.05$).
[f]Two mice were killed on day 22 due to severe ulceration at the tumor site even though the tumor was less than 1 g.

ious treatment groups were evaluated on the basis of percent increase in mean tumor weight, 30-day survival and complete tumor remission (Table 2). A DOX dose of 3.25 mg/kg per injection in free or liposomal form yielded tumor weight increases that were statistically comparable to control groups and no occurrence of complete tumor remission. However, 7% and 19% 30-day survival was observed for free and liposomal DOX, respectively, at this dose level. Increasing the dose of free DOX to 6.5 mg/kg per injection resulted in an increase in antitumor activity as evidenced by a 76% reduction in the tumor growth on day 8 as well as 25% 30-day survival. In comparison, liposomal DOX administered at 6.5 mg/kg per injection effected an 89% decrease in the tumor growth on day 8, 67% 30-day survival and 11% complete tumor remission. Statistical analysis indicated that inhibition of tumor growth for liposomal DOX was superior to equivalent doses of free drug (Table 2).

The ability to increase the DOX dose from the MTD of 6.5 mg/kg per injection for free drug to 13.0 mg/kg per injection in the less toxic liposomal form resulted in a dramatic inhibition of the tumor growth (Fig. 1 and Table 2). In addition, this treatment yielded a significant debulking of tumor load. For example, tumors reaching 3.4 g to 5.7 g within 8 days post first injection were reduced to less than 0.5 g over the full course of treatment (Fig. 1). Furthermore, 81% 30-day survival and 25% complete tumor regression indicated a substantial improvement of antitumor efficacy.

Discussion

The utility of carrier systems to enhance the therapeutic activity of anticancer agents can be accomplished by reducing drug-related toxicities to normal tissues, thereby allowing increased drug doses to be employed and/or by enhancing the antitumor potency of the drug. The present study suggests that the enhanced antitumor activity observed for liposomal DOX preparations utilized here is related to the ability of these carrier systems to increase tumor drug levels over an extended period of time. This improved delivery of DOX to the tumor occurs at doses equivalent to free drug as well as at doses greater than free drug. The former result contrasts another study employing a rat immunocytoma solid tumor

model [22] which demonstrated that liposome encapsulation results in reduced or equivalent DOX tumor uptake. One previous investigation has shown that increased antitumor activity of small (< 100 nm) liposomal DOX preparations correlates with increased drug levels in tumor cells [7]. However, in this tumor model the disease site resides in the liver, a known site of liposome accumulation. The results reported here provide clear indications that appropriately designed liposomal DOX systems [13] can be utilized to increase drug delivery to and activity against peripheral solid tumors.

Previous studies have demonstrated that small liposomes composed of suitable lipids exhibit extended circulation times and increased uptake into solid tumors [9]. This compares favorably with observations here where tumor liposome levels increased over 24 h, leading to approximately 5% of the administered liposome dose accumulating in the tumor. The results presented here suggest that such 'passive targeting' may lead to increased drug tumor levels. It should be noted, however, that plasma and tumor drug to lipid ratios indicate that a portion of initial tumor drug levels arises from DOX which has been released from liposomes in the circulation.

In summary, this study has demonstrated that liposomes exhibiting suitable physical properties can be used to increase the delivery of DOX to solid tumors. More importantly, such increases result in enhanced antitumor efficacy compared to that achievable with free DOX. The combination of increased tumor drug delivery and increased tolerated drug doses for liposomal DOX results in decreased tumor growth rate and reduction of tumor burden.

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EXHIBIT 5

DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 15(10), 1523-1554 (1989)

LIPOSOMES AS A DRUG DELIVERY SYSTEM

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INTRODUCTION

Liposomes have shown great potential as a drug delivery system. An assortment of molecules, including peptides and proteins, have been incorporated in liposomes, which can then be administered by different routes. Various amphiphathic molecules have been used to form the liposomes, and the method of preparation can be tailored to control their size and morphology. Drug molecules can either be encapsulated in the aqueous space or intercalated into the lipid bilayer; the exact location of a drug in the liposome will depend upon its physicochemical characteristics and the composition of the lipids.

Due to their high degree of biocompatibility, liposomes were initially conceived of as delivery systems for intravenous delivery. It has since become apparent that liposomes can also be useful for delivery of drugs by other routes of administration. The formulator can use strategies to design liposomes for specific purposes, thereby improving the therapeutic index of a drug by increasing the percent of drug molecules that reach the target tissue, or alternatively, decreasing the percent of drug molecules that reach sites of toxicity. Clinical trials now underway utilize liposomes to achieve a variety of therapeutic objectives including enhancing the activity and reducing toxicity of a widely used antineoplastic drug (doxorubicin) and an antifungal drug (amphotericin B) delivered intravenously. Other clinical trials are evaluating the ability of liposomes to deliver intravenously immunomodulators (MTP-PE) to macrophages and imaging agents (^{111}Indium) to tumors. Recent studies in animals have reported the delivery of water-insoluble drugs into the eye, and the prolonged release of an immunomodulator (interferon) and a peptide hormone (calcitonin) from an intramuscular depot. These trials and animals studies provide evidence of the versatility of liposomes.

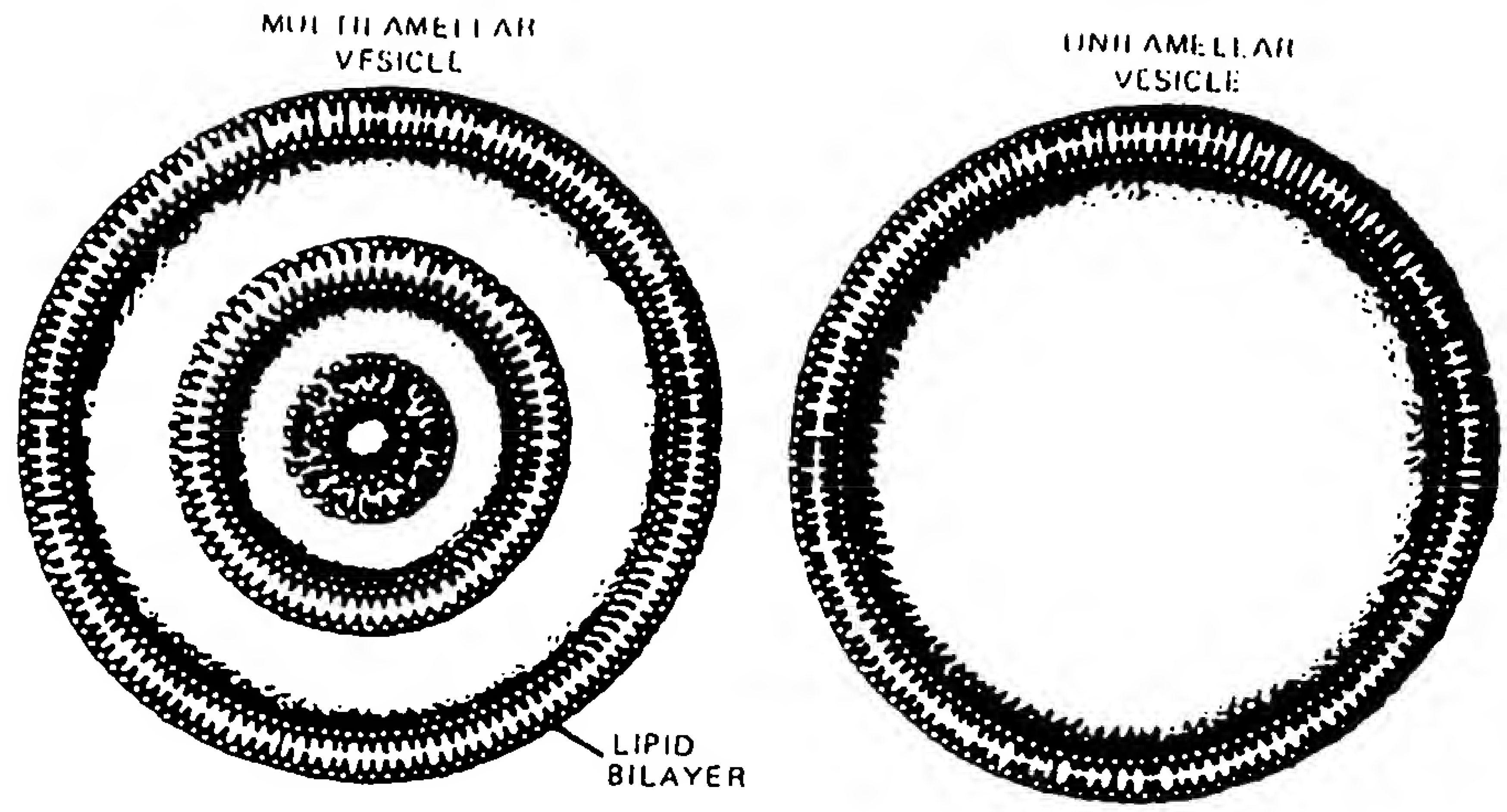

FIGURE 1
Diagramatic representation of multilamellar and unilamellar vesicles. Source: Ostro, M.J. (1987) Scientific American, 102-111.

A liposome is defined as a structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments (Fig. 1). These spherical structures can be prepared with diameters ranging from 80 nm to 100 μm. When phospholipids are dispersed in an aqueous phase, hydration of the polar head groups of the lipid results in a heterogeneous mixture of structures, generally referred to as vesicles, most of which contain multiple lipid bilayers forming concentric spherical shells. These were the liposomes first described by Bangham[1] and are now referred to as multilamellar vesicles (MLVs). Sonication of these lipid dispersions results in size reduction of these liposomes to vesicles containing only a single bilayer with diameters ranging from 25-50 nm. These structures are referred to as small unilamellar vesicles (SUVs). Since MLVs and SUVs have certain limitations as model membrane systems and as drug delivery systems, a number of laboratories have developed single bilayer liposomes which exhibit a size range of 100-500 nm in diameter. These vesicles are referred to as large unilamellar vesicles (LUVs). The nomenclature describing liposomes can be confusing since liposomes have been classified as a function of the number of bilayers (e.g., MLV, SUV), or as a function of the method of preparation (e.g., REV, FPV, EIV) or as a function of size (e.g., LUV, SUV). The following are examples of frequently used nomenclatures:

TABLE 1

Examples of Nomenclature Used to Describe Liposomes.

TYPE OF VESICLE	TERM USED	APPROX. SIZE (μm)
Small, Sonicated Unilamellar	SUV	0.025-0.05
Large, Vortexed Multilamellar	MLV	0.05-10
Large Unilamellar	LUV	0.1
Reverse Phase Evaporation	REV	0.5
French Press	FPV	0.05
Ether Injection	EIV	0.02

Materials Used in Liposome Preparation

The lipids most commonly used to prepare liposomes are shown in Fig. 2.

Phospholipids: Glycerol containing phospholipids are by far the most commonly used component of liposome formulations and represent more than 50% of the weight of lipid present in biological membranes. The general chemical structure of these types of lipids is exemplified by phosphatidic acid. The "backbone" of the molecule resides in the glycerol moiety. At position number 3 of the glycerol molecule the hydroxyl is esterified to phosphoric acid (hence the name glycerolphospholipids). The hydroxyls at positions 1 and 2 are usually esterified with long chain fatty acids giving rise to the lipidic nature of these molecules. One of the remaining oxygens of phosphoric acids may be further esterified to a wide range of organic alcohols including glycerol, choline, ethanolamine, serine and inositol. The phosphate moiety of phosphatidic acid carries a double negative charge only at high pH. The pK values for the two oxygens are 3 and about 7. At physiologically relevant pH values this molecule presents more than one net negative charge, but not quite 2. The most abundant glycerol phosphatides in plants and animals are phosphatidylcholine (PC), also called lecithin, and phosphatidylethanolamine (PE), sometimes referred to as cephalin. These two phosphatides constitute the major structural component of most biological membranes. In phosphatidylserine (PS),the phosphoric acid moiety of phosphatidic acid (PA) is esterified to the hydroxyl group of the amino acid L-serine, and in phosphatidylinositol (PI) to one of the hydroxyls of the cyclic sugar alcohol inositol. In the case of phosphatidylglycerol (PG), the alcohol that is esterified to the phosphate moiety is glycerol. Table 2 shows the fatty acid composition of two common phosphatidylcholines, one extracted from egg yolk and the other from soy bean oil. Notice the difference in the degree of unsaturation between egg and soy PC. Soy PC contains a greater proportion of unsaturated bonds and is thus more susceptible to peroxidation.

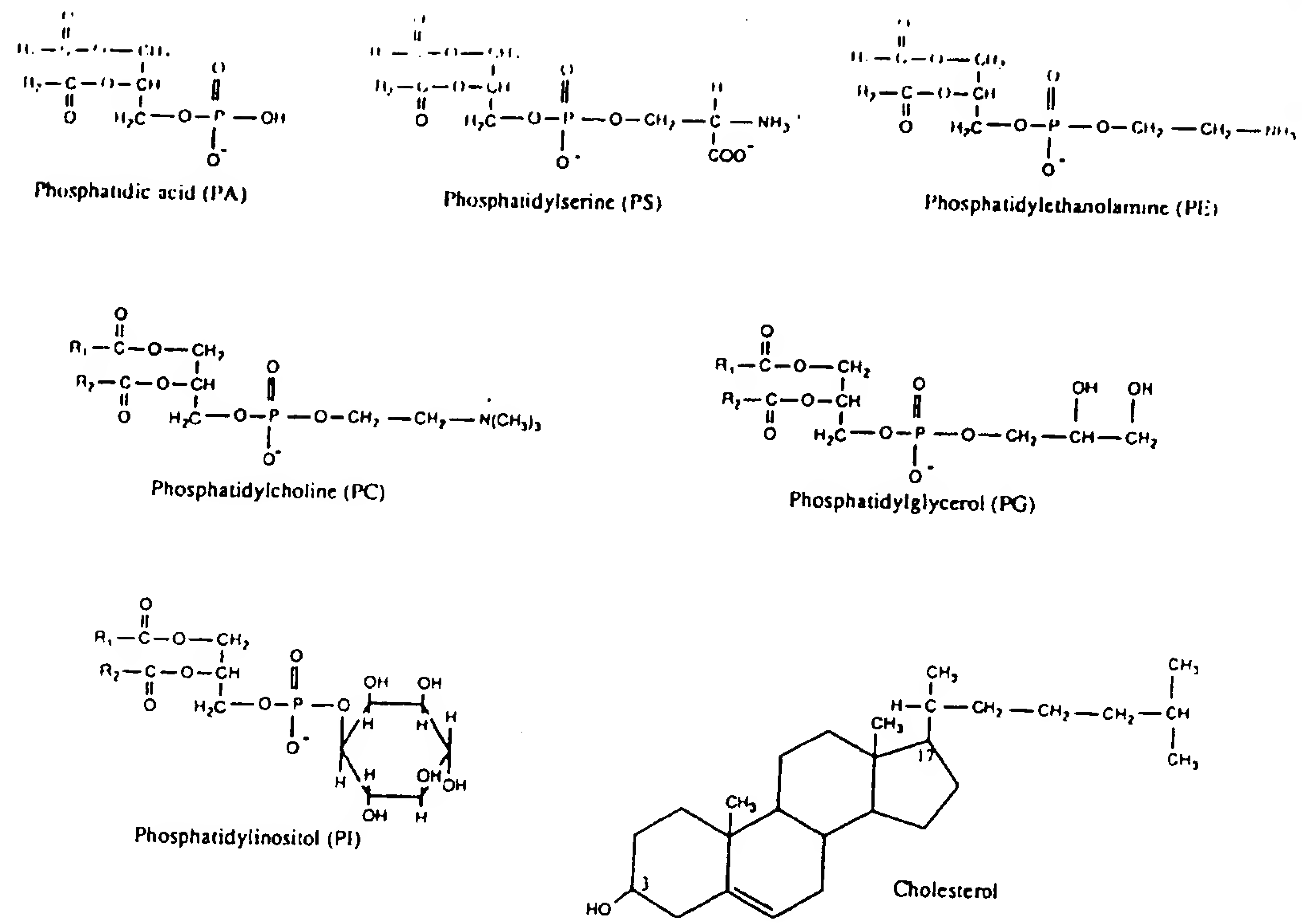

FIGURE 2
Chemical structures of lipids commonly used to prepare liposomes.

TABLE 2
Fatty acid composition of two common phosphatidylcholines, one extracted from egg yolk and the other from soy bean oil.

Fatty Acid Composition		Egg PC	Soy PC
16:0	Palmitic	32	12
16:1	Palmitoleic	1.5	<0.2
18:0	Stearic	16	2.3
18:1	Oleic	26	10
18:2	Linoleic	13	68
18:3	Linolenic	<0.3	5
20:4	Arachidonic	4.8	<0.1
22:6	Dodosapentaenoic	4.0	<0.1

Steroids: The steroid cholesterol and its derivatives are quite often included as components of liposomal membranes. Cholesterol is abundant in animal tissues and is primarily localized in cell membranes. Its inclusion in liposomal membranes has three recognized effects: (i) increasing the fluidity or microviscosity of the bilayer; (ii) reducing the permeability of the membrane to water soluble molecules; and (iii) stabilizing the membrane in the presence of biological fluids such as plasma. This latter effect has proven useful in formulating liposomes for drug delivery applications which use the intravenous route of administration. Liposomes without cholesterol are known to interact rapidly with plasma proteins such as albumin, transferrin and macroglobulins. These proteins tend to extract bulk phospholipids from liposomes thereby depleting the outer monolayer of the vesicles leading to physical instability. Cholesterol appears to substantially reduce this type of interaction. Cholesterol has been called the "mortar" of bilayers because, by virtue of its molecular shape and solubility properties, it fills in empty spaces among the phospholipid molecules, anchoring them more strongly into the structure.

Metabolic Fate of Bilayer Forming Lipids: An attribute of liposomes that translates into unusually high *in vivo* tolerance is the fact that the structural components of the system, phospholipids and cholesterol, are treated no differently than biological membrane lipids. In the body they are broken down by enzyme systems into natural intermediates like glycerol phosphate, fatty acids, ethanolamine, choline and acyl-Co-A and either metabolized further to provide energy, or enter a lipid pools which is drawn upon to build new lipids which replace those that naturally turn over in biological membranes. Phospholipids are hydrolyzed *in vivo* by specific phospholipases (Fig. 3). Phospholipases can be used *in vitro* to modify natural lipids. For example, phospholipase A-2 isolated from snake venom has been used to produce lysophosphatidylcholine from natural phosphatidylcholine. Phospholipase D is being used commercially to produce "semisynthetic" PS, PA and PG from PC. PC dissolved in ether is added and the two phases emulsified. The enzyme catalyzes head group exchange. The rates and yields of the conversion are dependent on the activity of the enzyme and the molar excess of the alcohol to be exchanged for choline

The liver serves both as the chief source and chief organ for the disposal of cholesterol. A major portion of the cholesterol removed from plasma lipoproteins by the liver is excreted in the bile.

Synthetic Phospholipids: Generally used saturated phospholipids include dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylserine (DPPS), dipalmitoylphosphatidic acid (DPPA) and dipalmitoylphosphatidylglycerol (DPPG). Several unsaturated phospholipids have also been used for preparing liposomes; these include dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG).

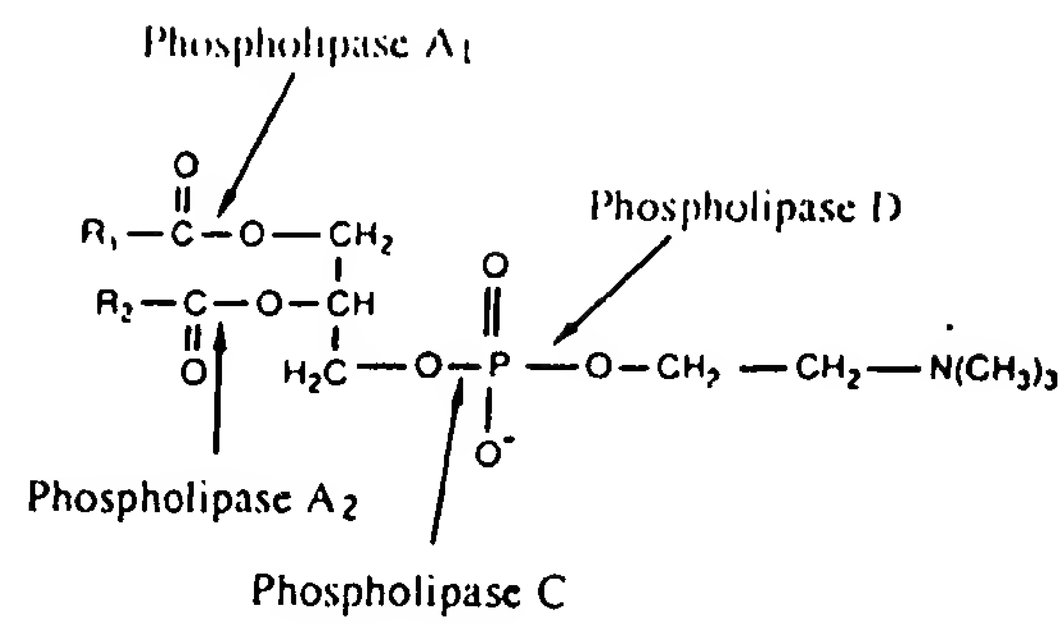

FIGURE 3
Sites of action of phospholipases on phosphatidylcholine.

Other Substances: Diacylglycerol, stearylamine and dicetylphosphate have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Also, a number of compounds having a single long-chain hydrocarbon and an ionic head group has been synthesized and found to be capable of forming vesicles. These include quaternary ammonium salts and dialkyl phosphates.

Lipid Selection: Many liposome-based pharmaceutical products are entering the clinical trial stage of development; several of these may reach the marketplace in a few years. The lipid component of these products must meet stringent "pharmaceutical" requirements in order to obtain regulatory approval for large-scale human testing and marketing. These include suitable purity, safety and microbial/endotoxin limits, and adequate stability. Currently-available pharmaceutical grade lipid products (such as egg and soy phosphatides) were developed primarily for the parenteral emulsions industry and in general they are not well suited for liposome formulations. Liposomes composed of crude egg yolk phosphatides, for example, are not stable at ambient temperatures for more than a few months. Thus developers of liposome products have been relying upon specialty chemical firms to supply highly purified lipids for their raw material needs. The current cost for these high purity lipids will need to be addressed for large scale production and commercialization of liposome products. Although liposome-based products are within reach, and their market potential is large, successful commercialization depends in part on the willingness of lipid suppliers to differentiate their product lines in response to the needs of the rapidly emerging liposome industry. Since each liposome-based product has its unique stability, safety and purity requirements, it is likely that a range of lipids including natural products, semi-synthetics and synthetics of varying degrees of purity will be needed. The key pharmaceutical and commercial issues that remain to be addressed by both the lipid

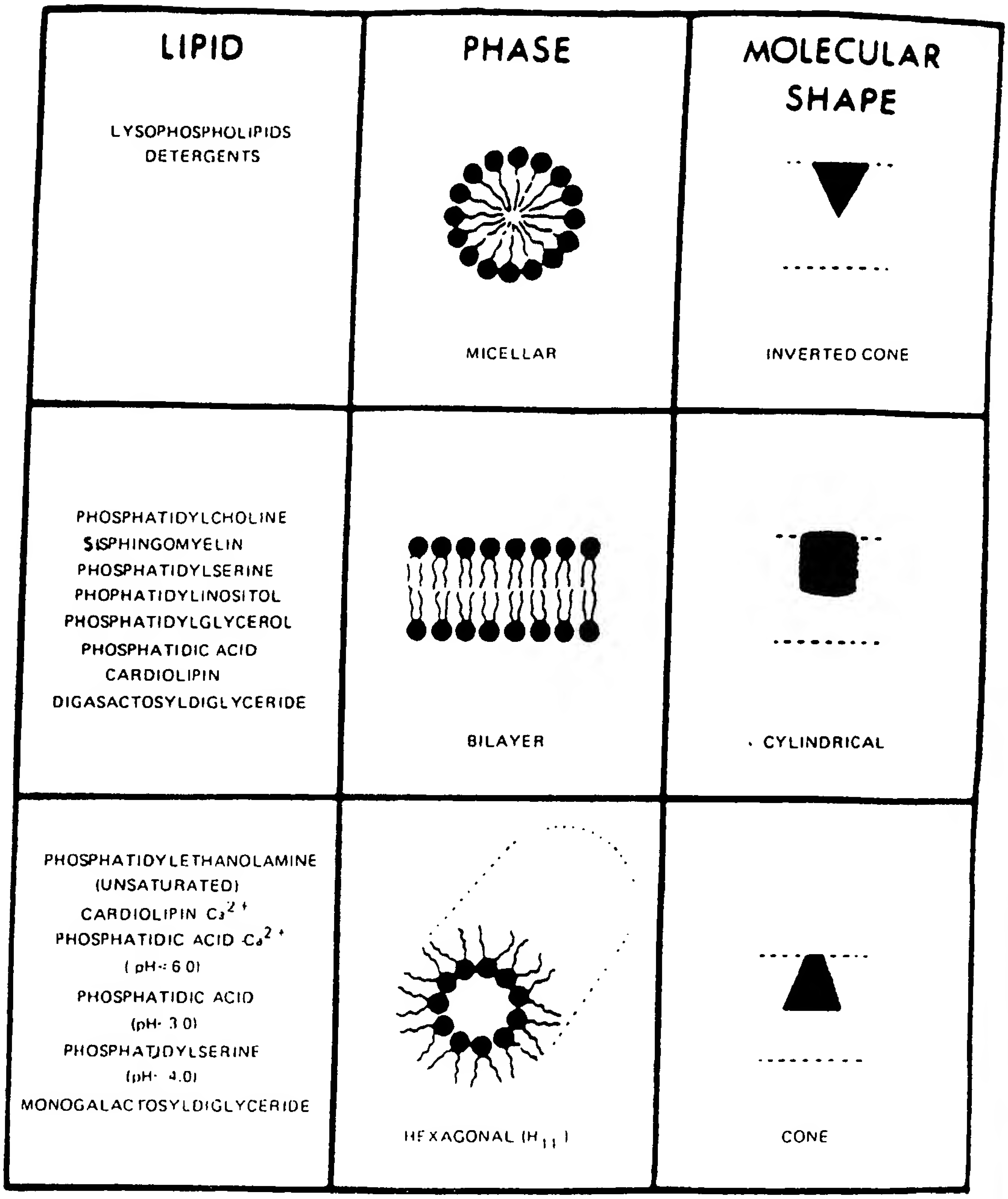

FIGURE 4
Effect of molecular geometry on phase properties of lipids. Source: Cullis, P.R. and Hope, M.J. (1985) "Physical Properties and Functional Roles of Lipids" in *Biochemistry of Lipids and Membranes*, Vance, D.E. and Vance, J.E., eds., Benjamin/Cummings Inc., p. 56.

suppliers and liposome product developers include the following:

a. Quantities, purities and pharmaceutical attributes of lipids required for liposome products;
b. Detailed specifications for each lipid, including standardized nomenclature and analytical quality control procedures;
c. Introduction of the above into official compendia; and
d. Expansion/centralization of a data base on the stability and safety of key lipids.

WHY LIPOSOMES ARE FORMED

Lipids capable of forming liposomes (or other colloidal structures) exhibits a dual chemical nature. Their head groups are hydrophilic (water loving) and their fatty acyl chains are hydrophobic (water hating). It has been estimated that each zwitterionic head group of phosphatidylcholine has on the order of 15 molecules of water weakly bound to it, which explains its overwhelming preference for the water phase. The hydrocarbon fatty acid chains, on the other hand, vastly prefer each other's company to that of water. This phenomenon can be understood in quantitative terms by considering the critical micelle concentration (c.m.c.) of PC in water. The c.m.c. is defined as the concentration of the lipid in water (usually expressed as moles per liter) above which the lipid forms either micelles or bilayer structures rather than remaining in solution as monomers. The c.m.c. of dipalmitoylphosphatidylcholine has been measured by Smith and Tanford[2] and found to be 4.6×10^{-10} M in water. This value is in agreement with those obtained for similar amphiphiles. Clearly, this is a very small number indicating the overwhelming preference of this molecule for a hydrophobic environment such as that found in the core of a micelle or bilayer.

The large free energy change between a water and a hydrophobic environment ($\approx$15.3 Kcal/mole for dipalmitoylphosphatidylcholine and $\approx$13.0 Kcal/mole for dimyristoylphosphatidylcholine) explains the overwhelming preference of typical lipids to assemble in bilayer structures excluding water as much as possible from the hydrophobic core in order to achieve the lowest free energy level and hence the highest stability for the aggregate structure. It is also clear from these thermodynamic considerations that bilayer structures do not exist as such in the absence of water because it is water that provides the driving force for lipid molecules to assume a bilayer configuration.

A high degree of surface activity of a given molecule does not guarantee it ability to form bilayer structures in the presence of water. The type of physical structure they attain under a given set of conditions will depend on their interactions with neighboring molecules, their interaction with water and most importantly, whether the surface area of the polar head group, upon hydration, is smaller or larger than the surface area of the hydrophobic group (Fig. 4). For example, phosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol and phosphatidylglycerol have a preference for bilayer structures (liposomes). On the other hand, lysophospholipids form micelles

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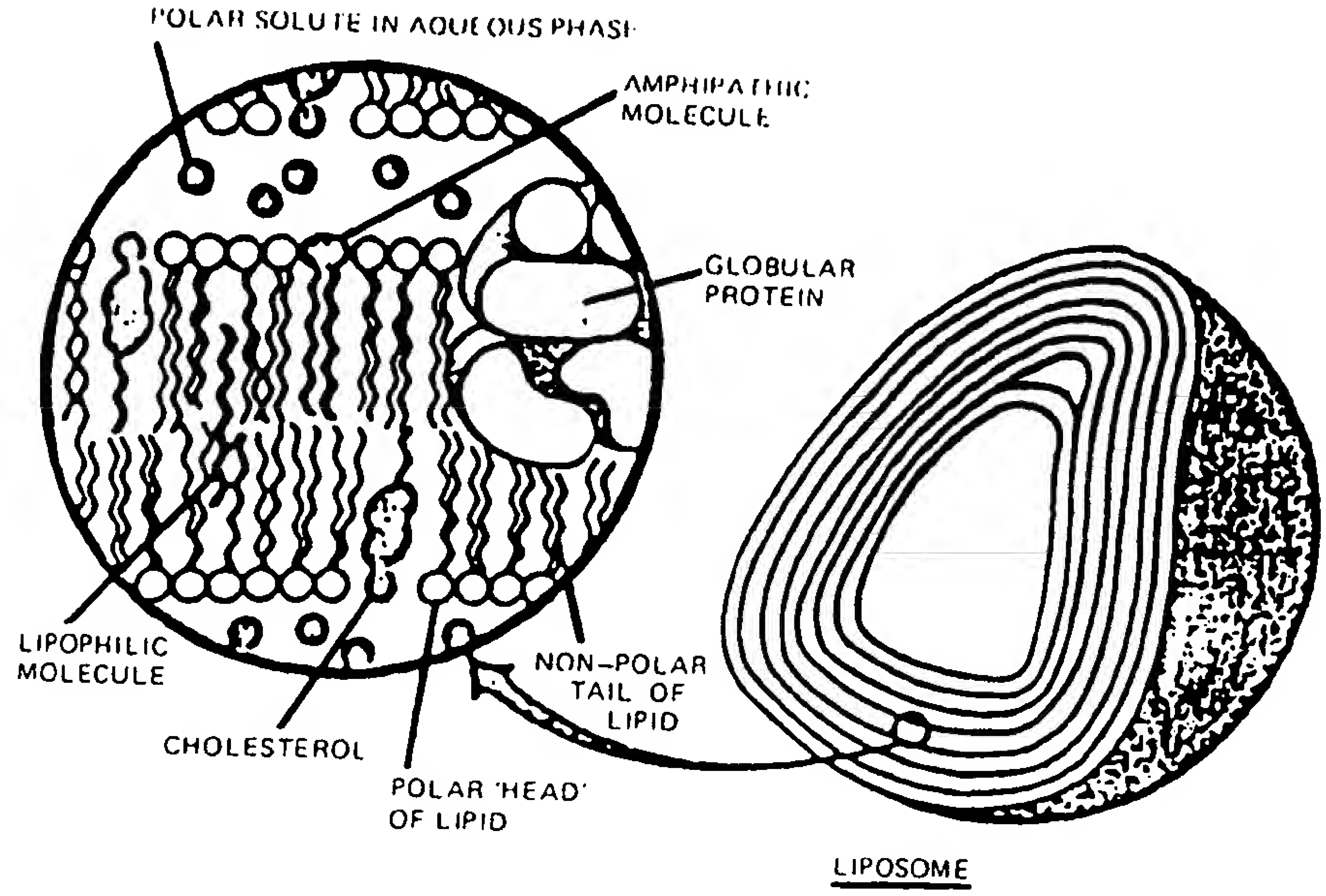

FIGURE 5
Diagrammatic representation the various sites in the liposome available for drug entrapment. Source: Gregoriadis, G. and Allison, A.C. (1980) *Liposomes in Biological Systems,* John Wiley & Sons, Ltd. New York, p. 89.

and phosphatidylethanolamines and negatively charged phospholipids under certain conditions (low pH and in the presence of divalent cations) form hexagonal (H_{II}) structures. It should be pointed out that under proper conditions, relatively large amounts of lipids which normally tend to form hexagonal or micellar structures can be successfully incorporated into liposomes.

CHARACTERIZATION OF LIPOSOMES

Factors Affecting Drug Entrapment: The amount and location of a drug within a liposome is dependent on a number of factors. The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayers, and the maximum amount of drug that can be entrapped within a liposome is dependent on its total solubility in each phase. For example, very little 6-mercaptopurine can be encapsulated in liposomes because this drug has limited solubility in both polar and non-polar solvents. The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of non-polar

and polar drug, respectively, that can be loaded into a liposome. Efficient capture will depend on the use of drugs at concentrations which do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for non-polar drugs). The method of preparation can also affect drug location and overall trapping efficiency. Fig. 5 diagrammatically represents the various sites in the liposome available for drug entrapment.

Incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer) may be undesirable. If liposomes are prepared by mixing the drug with the lipids, the drug will eventually partition to an extent depending on the partition coefficient of the drug and the phase volume ratio of water to bilayer. Also, the rate of partitioning will be a function of its diffusivity in each phase. Release rates (a measure of instability) are highest when the drug has an intermediate partition coefficient. Bilayer/aqueous compartment partition coefficients are usually estimated by determining their organic solvent/water (e.g., octanol/water) partition coefficients. They can also be determined precisely by a method described by Bakouche and Gerlier[3] which is based on the physical separation of the aqueous and bilayer phases by ultracentrifugation after mechanical (ultrasonics at low temperatures) disruption of the liposomes followed by analysis of each phase for drug.

Internal Volume and Encapsulation Efficiency: These two parameters are used to describe entrapment of water soluble drugs in the aqueous compartments of liposomes. The internal or trapped or capture volume is expressed as aqueous entrapped volume per unit quantity of lipid (µl/µmol or µl/mg). It is determined by entrapping a water soluble-marker such as 6-carboxyfluorescein, ^{14}C or ^{3}H-glucose or sucrose and then lysing the liposomes by the use of a detergent such as Triton X-100. Determination of the amount of marker that was trapped enables one to back-calculate the volume of entrapped water. The encapsulation efficiency describes the percent of the aqueous phase (and hence the percent of water-soluble drug) that becomes entrapped during liposome preparation. The remaining drug remains outside of the liposome and is therefore "wasted". Encapsulation efficiency is usually expressed as % entrapment/mg lipid.

The internal or trapped volume and encapsulation efficiency greatly depends on liposomal content, lipid concentration, method of preparation and drug used. Some typical values are:

Liposome Type	Internal Volume µl /µmol lipid	Entrapment Efficiency %/mg lipid
SUV	<0.5	<1
MLV	>4	5-15
REV	>10	35-65

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Incorporation of charged lipids into bilayers increases the volume of the aqueous compartments by separating adjacent bilayers due to charge repulsion resulting in increases in trapped volume It should be pointed out that for hydrophobic drugs, entrapment efficiency usually approaches 100% almost irrespective of liposomal type and composition.

Lamellarity: The average number of bilayers present in liposomes can be found by freeze-fracture electron microscopy and ^{31}P-NMR. In the latter technique, the signals are recorded before and after the addition of nonpermeable broadening agent such as Mn^{2+}. Manganese ions interact with the outer leaflet of the outermost bilayer. Thus, a 50% reduction in NMR signal means that the liposome preparation is unilamellar and 25% reduction in the intensity of the original NMR signal means there are 2 bilayers in the liposomes[4].

Size and Size Distribution: The average size and size distribution of liposomes are important parameters with respect to physical properties and biological fate of the liposomes and their entrapped substances. There are a number of methods used to determine this parameter, but the most commonly used methods are:

a. Light Scattering: There are a variety of techniques available to size liposomes based on light scattering. The popularity of this method depends on its ease of operation and the speed by which one can obtain data. The newer instruments are based on dynamic laser light scattering.

If the liposomes to be analyzed were monodisperse, light scattering would be the method of choice; unfortunately, most preparations are heterogeneous, and they require an accurate estimation of their size-frequency distributions. Light scattering methods rely on algorithms to determine particle size distributions and the results obtained can be very misleading. Some complex algorithms have been developed in an attempt to deal with this problem. Furthermore, such methods can not distinguish between a large particle and a flocculated mass of smaller particles. Most importantly, it may is necessary to remove any micron-sized particles that are present in the sample prior to analysis.

The difficulty in interpreting particle size data can be demonstrated by taking a simple example of a dispersion comprised of 97% unilamellar vesicles with a radius of 15 nm and 3% multilamellar vesicles with a radius three times greater (45 nm):

	15 nm Particles	45 nm Particles
Percent Particles	97	3
Percent Surface Area	78	22
Percent Total Volume	54	46
True Statistical Average Radius	15.9 nm	
Instrumental Average Radius	25.3 nm	

Thus, 3% of the particles comprise almost one-half the volume of liposomes. Of course, the same problem of data analysis occurs with other disperse systems such as emulsions and suspensions.

b. Light Microscopy: This method can be used to examine the gross size distribution of large vesicle preparations such as MLVs. The inclusion of a fluorescent probe in the bilayer permits examination of liposomes under a fluorescent microscope and is a very convenient method to obtain an estimate of at least the upper end of the size distribution.

c. Negative Stain Electron Microscopy: This method, utilizing either molybdate or phosphotungstate as a stain is the method of choice for size distribution analysis of any size below 5 µm. It should be used to validate light scattering data that will ultimately be used for quality assurance. For accurate statistical evaluation (±5%), one should count at least 400 particles and not rely on a single specimen for counting.

d. Freeze Fracture Electron Microscopy: This method is especially useful for observing the morphological structure of liposomes. Since the fracture plane passes through vesicles that are randomly positioned in the frozen section, resulting in nonmidplane fractures, the observed profile diameter depends on the distance of the vesicle center from the plane of the fracture. Mathematical methods have been devised to correct for this effect.

For all of the microscopy procedures used, one should always be on the lookout for aggregated particles or flocs.

Application of Double Layer Theory to Liposomes: Once assembled, liposomes behave in much the same way as other charged colloidal particles suspended in water or electrolyte solutions. Under conditions where the charge on each particle is weak, the electrostatic repulsive force among the particles is also weak, increasing the opportunity for close approach. Some neutral particles tend either to flocculate or aggregate and sediment from suspension for this reason. Similarly, two populations of liposomes bearing opposite electric charges will aggregate at a rate that is a function of the electrostatic attractive forces among the particles. Particles bearing net negative charges may be induced to aggregate strongly in the presence of di- or trivalent cations. For example, calcium in the 1-2 mM range will induce liposomes containing more than 50 mole% PS to aggregate. These phenomena have dramatic effects on the physical stability of liposomes and lead to fusion of liposomes with one another resulting in increases in their overall size. Like aggregation, particle size growth, particularly during storage, would be undesirable in most products. Fortunately the tendency of liposomes to aggregate and fuse can be controlled by the inclusion of small amounts of negatively charged lipids such as PS or PG or positively charged amphiphiles such as stearylamine in the formulation. Knowing the number and the sign of charged groups added and the valency and concentration of electrolytes in the

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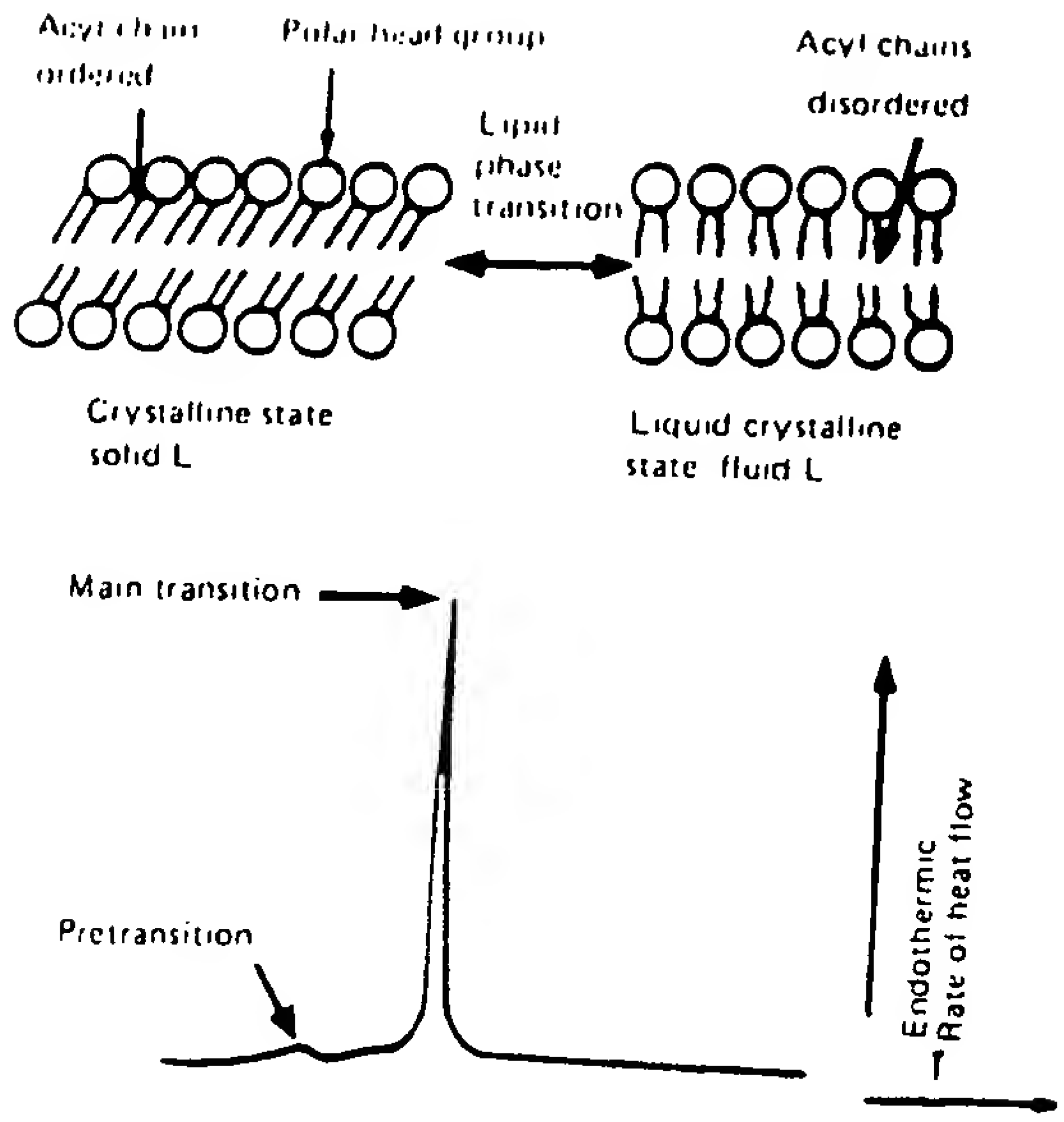

FIGURE 6
Phospholipid gel to liquid crystalline phase transition. Source: Cullis, P. R. and Hope, M. J. (1985) "Physical Properties and Functional Roles of Lipids in Membranes", In *Biochemistry of Lipids and Membranes* Vance, D. E. and Vance, J. E., Ed., The Benjamin/Cummings Publishing Co., California, p. 43.

medium, the magnitude of the electrostatic forces generated by these charged groups can be closely approximated using double layer theory. These results can then be correlated with physical stability of liposomes and used to guide formulation efforts. The amount of charged component and ionic conditions in a particular liposome dosage form can be adjusted to produce a high enough zeta potential to inhibit close approach of the vesicles and prevent aggregation. In practice it is usually necessary to determine empirically the magnitude of the zeta potential required to prevent aggregation in a particular system. However, once this has been done, it is possible to use the zeta potential as a quality control check to insure that each batch of liposomes contains sufficient charged groups to avoid aggregation during storage.

Phase Behavior of Liposomes: An important feature of membrane lipids is the existence of a temperature-dependent reversible phase transition, where the hydrocarbon chains of the phospholipid undergo a transformation from an ordered (gel) state to a more disordered fluid (liquid crystalline) state. These changes have been documented by freeze-fracture electron microscopy but are most easily demonstrated by differential scanning calorimetry (DSC). Fig. 6 illustrates the phase transition region for a typical phospholipid.

TABLE 3
Phase transition temperatures of some synthetic phospholipids used to prepare liposomes.

LIPID	CHARGE	Tm (°C)
Dilauryl Phosphatidylcholine	0	0
Dimyristoyl Phosphatidylcholine	0	23
Dipalmitoyl Phosphatidylcholine	0	41
Dimyristoyl Phosphatidylethanolamine	0	48
Distearoyl Phosphatidylcholine	0	58
Dipalmitoyl Phosphatidylethanolamine	0	60
Dioleoyl Phosphatidylglycerol	-1	-18
Dilauryl Phosphatidylglycerol	-1	4
Dimyristoyl Phosphatidylglycerol	-1	23
Dipalmitoyl Phosphatidylglycerol	-1	41
Distearoyl Phosphatidylglycerol	-1	55

The physical state of the bilayer profoundly affects the permeability, leakage rates and overall stability of the liposomes. The phase transition temperature (T_m) is a function of the phospholipid content of the bilayer.

By proper admixture of bilayer forming materials, one may design liposomes to "melt" at any reasonable temperature. This strategy has been used to deliver methotrexate to solid tumors which are heated to the phase transition temperature of the custom designed liposomal phospholipids. The phase transition temperature can be altered by using phospholipid mixtures or by adding sterols such as cholesterol. The T_m value can give good clues as to liposomal stability and permeability and as to whether a drug is entrapped in the bilayer or the aqueous compartment.

Liposome Preparation Methods

Multilamellar Vesicles (MLV): Multilamellar vesicles are by far the most widely studied type of liposome and, as pointed out by Alec Bangham in 1974, exceptionally simple to make. In general a mixture of lipids is deposited as a thin film on the bottom of a round-bottom flask by rotary evaporation under reduced pressure. MLVs form spontaneously when an excess volume of aqueous buffer is added to the dry lipid. However, in many cases MLVs have not been rigorously characterized with respect to size, polydispersity, number of lamellae, encapsulated volume and stability. Due to their ease of production many investigators have simply made a preparation of MLVs for use in both *in vitro* and *in vivo* experiments without taking the time to fully characterize them. This has led to a great deal of confusion in the interpretation of experimental results because, as will be explained below, minor changes in the method of preparation can lead to major differences in the behavior of liposomes.

Slow vs. Fast Hydration, Thickness of the Lipid Film: The time allowed for hydration and conditions of agitation are critical in determining the amount of the aqueous buffer (or drug solution) entrapped within the internal compartments of the MLV. For example, as pointed out by Szoka and Papahadjopoulos[5], a similar lipid concentration can encapsulate 50% more of the aqueous buffer per mole of lipid when hydrated for 20 hours with gentle shaking, compared to a hydration period of 2 hours with vigorous shaking, despite the fact that the two preparations exhibit a roughly similar particle size distribution. If hydration time is reduced to a few minutes with vortexing, a suspension will exhibit a still lower capture volume and a smaller mean diameter. As pointed out by Bangham[1], the hydration and entrapping process is most efficient when the film of dry lipid is kept thin. This means that different sized round-bottom flasks should be used for different quantities of lipid. Glass beads have been used by some investigators to increase the surface area available for film deposition. Thus the hydration time, method of suspension of the lipids and the thickness of the film can result in markedly different preparations of MLVs, in spite of identical lipid concentrations and compositions, and volume of the suspending aqueous phase.

Effect of Charged Lipids: The presence of negatively charged lipids such PS, PA, PI or PG, or positively charged detergents such as stearylamine will tend to increase the interlamellar distance between successive bilayers in the MLV structure and thus lead to a greater overall entrapped volume. This is particularly true in low ionic strength buffers or non-electrolytes (such as sucrose) since the electrostatic repulsive forces which give rise to the effect are greater under these conditions. Generally about 10-20 mole percent of a charged species is used although it is possible to produce MLVs from a purely charged lipid such as PS. The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs.

Hydration in the Presence of Solvent: MLVs with high entrapment of solutes can be produced by hydrating the lipid in the presence of organic solvents. A method introduced by Papahadjopoulos[6] begins with a two-phase system consisting of equal volumes of petroleum ether containing bilayer forming lipids and aqueous phase. The contents of the tube are emulsified by vigorous vortexing and the ether removed by passing a stream of nitrogen gas over the mixture. As the ether is removed in the carrier gas, MLVs form in the aqueous phase. A similar method was reported by Gruner et al.[7] except that diethyl ether was used as the solvent, sonication was used in place of vortexing and the aqueous phase was reduced to a relatively small proportion. Typically the lipids are dissolved in about 5 ml ether and about 0.3 ml of the aqueous phase to be entrapped is added and the two phases are emulsified by sonication while a gentle stream of nitrogen gas is passed over the mixture. The resulting MLV preparation encapsulates up to 40% of the solvent throughout the hydration step, and the concentration of solute molecules is in equilibrium across all the bilayers, a feature that is claimed to translate into greater stability to leakage.

MLVs Formed by Freeze Drying SUV Dispersions: A simple method for preparing MLVs with high entrapment efficiency was developed by Ohsawa et al.[8] and Kirby and Gregoriadis[9]. The aqueous phase containing the molecules to be encapsulated is mixed with a preformed suspension of SUVs and the mixture freeze dried by conventional means. Large MLVs are formed when the dry lipid is rehydrated, usually with a small volume of distilled water. Encapsulation efficiencies up to 40% have been reported for this method.

Small Unilamellar Vesicles (SUV): The classical methods of dispersing phospholipids in water to form optically clear suspensions with a particle weight of about 2×10^6 daltons involve various mechanical means and began with the sonication method reported in the mid 60's[10] followed by refinements introduced by Hamilton and his colleagues[11] and Barenholz[12] in the mid 70's who employed a high pressure device to produce the same effect in larger volumes. These types of SUV dispersions have been rigorously characterized by Huang[13] and others and shown to consist of rather uniform closed bilayer vesicles of about 25-50 nm diameter. Solvent injection methods have also been devised to produce SUVs. These typically involve the slow injection of a lipid solution in either ethanol or ether into warm water containing a drug or other marker to be entrapped. All of these methods are discussed in greater detail below.

Sonicated SUVs: The preparation of sonicated SUVs has been reviewed in detail by Bangham[14]. Briefly, the usual MLV preparation is subsequently sonicated either with a bath type sonicator or a probe sonicator under an inert atmosphere (usually nitrogen or argon). Although probe sonication leads to more rapid size reduction of the MLVs, degradation of lipids, metal particle shedding from the probe tip and aerosol generation can present problems. Bath type sonicators also have disadvantages (such as the need to pay greater attention to position of the tube and water level in the bath) but temperature can be accurately regulated. Also, the tube containing the specimen is sealed allowing for aseptic operations and little likelihood of personnel exposure to aerosols.

French Pressure Cell: Dispersions of MLVs can be converted to SUVs by passage through a small orifice under high pressure. A French Pressure Cell was used by Hamilton et al.[15] for this purpose. MLV dispersions are placed in the French Press and extruded at about 20,000 psi at 4°C. One pass through the cell produces a heterogeneous population of vesicles ranging from several microns in diameter to SUV size. Multiple extrusions results in a progressive decrease in the mean particle diameter. Following about 4-5 passes, about 95% of the vesicles have converted to SUVs as judged by size exclusion chromatography. The resulting vesicles are somewhat larger than sonicated SUVs ranging in size from 315-500 A. The method is simple, reproducible and nondestructive. However, temperature control is difficult (the pressure cell must be allowed to cool between extrusions or the temperature rise may

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damage the lipids) and the working volumes are relatively small (about 50 ml maximum).

Solvent Injection Method

a. Ether Infusion: A method introduced by Deamer and Bangham in 1976[16] provides a means of making SUVs by slowly introducing a solution of lipids dissolved in diethyl ether (or ether/methanol mixtures) into warm water. Typically the lipid mixture is injected into an aqueous solution of the material to be encapsulated (using a syringe-type infusion pump) at 55-65°C or under reduced pressure. Subsequent removal of residual ether under vacuum leads to the formation of single layer vesicles. Depending on the condition used, the diameters of the resulting vesicles ranges from 50-200 nm. The usual lipid concentration is about 2 mg/ml ether and about 2 ml of this solution are infused into 4 ml of the aqueous phase at a rate of 0.2 ml/min. at 50-60° C.

b. Ethanol Injection: An alternative method for producing SUVs that avoids both sonication and exposure to high pressure is the ethanol injection technique described by Batzri and Korn[17]. Lipids dissolved in ethanol are rapidly injected into a vast excess of buffer solution forming SUVs spontaneously. The procedure is simple, rapid and avoids exposure of both lipids and the material to be entrapped to harsh conditions. Unfortunately, the method is restricted to the production of relatively dilute SUV suspensions. The final concentration of ethanol cannot exceed about 10% by volume or the SUVs will not form. Removal of residual ethanol can also present a problem since ethanol forms an azeotrope with water which is difficult to remove under vacuum or by distillation. Various available ultrafiltration apparatus may be used to both concentrate the suspension and remove ethanol, however, these procedures tend to be slow and expensive to scale up. Another limitation of the method is related to the susceptibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol.

Large Unilamellar Vesicles (LUV) and Intermediate Sized Unilamellar Vesicles: Large unilamellar vesicles provide a number of important advantages as compared to MLVs including high encapsulation of water soluble drugs, economy of lipid and reproducible drug release rates. However, LUVs are perhaps the most difficult type of liposome to produce. "Large" in the context of liposomes usually means any structure larger than 100 nm; thus large unilamellar vesicles refers to vesicles bounded by a single bilayer membrane that are above 100 nm in diameter. Some authors have referred to liposomes between the sizes of 50-100 nm as "large" but these would be more appropriately called intermediate sized. Two primary methods are used to produce LUVs, one involving detergent dialysis, the other a sophisticated reverse emulsification technique. Intermediate sized single layered vesicles can be generated from MLV dispersions by sequential extrusion through small pore size polycarbonate membranes under high pressure. A number of other techniques for producing LUVs have been reported including freeze thawing, slow swelling in nonelectrolytes,

dehydration followed by rehydration and dilution or dialysis of lipids in the presence of chaotropic ions. Each of these methods is reviewed below.

LUVs Formed by Detergent Removal: An essentially different approach to produce liposomes is dependent on the removal of detergent molecules from aqueous dispersions of phospholipid/detergent mixed micelles. As the detergent is removed the micelles become progressively richer in phospholipid and finally coalesce to form closed single bilayer vesicles. Three methods of detergent removal appropriate for this purpose have been described in the literature and are treated separately below.

a. Dialysis: Kagawa and Racker[18] were the first to introduce the dialysis method for lipid vesicle preparation. Although these authors were primarily interested in reconstituting biological membranes solubilized with detergents, their method is applicable to the formation of liposomes as well. Detergents commonly used for this purpose exhibit a reasonably high critical micelle concentration (on the order of 10-20 mM) in order to facilitate their removal and include the bile salts sodium cholate and sodium deoxycholate and synthetic detergents such as octylglucoside. The treatment of egg PC with a 2:1 molar ratio of sodium cholate followed by dialysis results in the formation of vesicles in the 100 nm diameter range within a few hours. Another modification of the cholate removal technique is one in which the rate of efflux of the detergent from the mixture is controlled. This procedure described in detail by Milsmann et al.[19] employs a phospholipid:detergent ratio of 0.625 and rapidly removes the detergent in a flow through dialysis cell. The procedure forms a homogeneous population of single layered vesicles with mean diameters of 50-100 nm. A commercial version of the dialysis system is available under the trade name LIPOPREP™.

b. Column Chromatography: The formation of 100 nm single layered phospholipid vesicles during removal of deoxycholate by column chromatography has been reported by Enoch and Strittmatter[20]. The method involves the treatment of phospholipid, in the form of either small sonicated vesicles or a dry lipid film, at a molar ratio of deoxycholate to phospholipid of 1:2. Subsequent removal of the detergent during passage of the dispersion over a Sephadex G-25 column results in the formation of uniform 100 nm vesicles that are readily separated from small sonicated vesicles.

c. Bio-beads™: Another promising method for forming reconstituted membranes reported by Gerritsen et al.[21] may also be applicable to LUV preparation. The system involves the removal of a nonionic detergent, Triton X-100, from detergent/phospholipid mixtures. This method is based on the ability of Bio-beads SM-2 to adsorb Triton X-100 selectively and rapidly. The dried lipid is suspended in 0.5-1.0 % Triton X-100 and washed Bio-beads are added directly to the solution (about 0.3 g wet Bio-beads per ml of dispersion) and rocked for about 2 hours at 4°C.

The beads are removed by filtration. The final particle size is determined by the conditions used including lipid composition, buffer composition, temperature and, most critically, the amount and activity of the beads themselves.

<u>Reverse Phase Evaporation Technique (REV)</u>: LUVs can also be prepared by forming a water-in-oil emulsion of phospholipids and buffer in excess organic phase followed by removal of the organic phase under reduced pressure (the so called "Reverse Phase Evaporation or REV method). The two phases are usually emulsified by sonication but other mechanical means have also been used. Removal of the organic solvent under vacuum causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent results in the collapse of the gel into a smooth suspension of LUVs. With some lipid compositions the transition from emulsion to LUV suspension is so rapid that the intermediate gel phase appears not to form. The method which was pioneered by Szoka and Papahadjopoulos in 1978[22] has been used extensively for applications which require high encapsulation of a water soluble drug. Entrapment efficiencies up to 65% can be obtained with this method. The phospholipids are first dissolved in an organic solvent such as diethylether, isopropylether or mixtures of two solvents such as isopropylether and chloroform. The emulsification is most easily accomplished when the density of the organic phase matches that of the buffer (i.e., about 1). For this reason, ether (density of about 0.7) is often mixed with a solvent of higher density such as trichlorotrifluoroethane (density of 1.4) to produce a solvent system with a density close to water. The aqueous phase containing the material to be entrapped is added directly to the phospholipid-solvent mixture. The ratio of aqueous phase to organic phase is usually about 1:3 for ether and 1:6 for isopropylether-chloroform mixtures. Preparations using even greater proportions of organic phase have been reported. The two phases are emulsified by sonication for a few minutes and the organic phase removed slowly under a partial vacuum produced by a water aspirator on a rotary evaporator at 20-30° C. The vacuum is usually maintained at about 500 microns for the first few minutes (using a nitrogen gas bleed to lower the vacuum and a gauge to measure the vacuum) and then raised cautiously to fill the aspirator vacuum to prevent the ether from evaporating too quickly. A typical preparation contains 60 µmol lipid dissolved in 3 ml ether and 1 ml aqueous phase contained in a sealed screw cap tube The mixture is sonicated in a bath type sonicator for about 5 minutes or until a homogeneous emulsion is formed. For a quick check to determine if emulsification is complete, one can interrupt sonication and allow the tube to stand for about a minute. If a clear layer of ether is observed over the aqueous phase, sonication should be continued for an additional period. Maximal encapsulation (65%) is obtained when the ionic strength of the aqueous phase is low. The method has been used to encapsulate both small and large molecules. Biologically active macromolecules such as RNA and various enzymes have been encapsulated without loss of activity. The principal disadvantage of the method is the exposure of the material to be

encapsulated to organic solvents and mechanical agitation, conditions that lead to the denaturation of some proteins or breakage of DNA stands.

Formation of Intermediate Sized Unilamellar Vesicles by High Pressure Extrusion: As mentioned above, MLV suspensions rich in acidic lipids such as PS or PG tend to have large interbilayer distances and large internal aqueous cores due to electrostatic repulsive forces among the bilayers. Hope, et al.[23] among others have shown that as MLVs are repeatedly extruded through very small pore diameter polycarbonate membranes (0.8-1.0 micron) under high pressure (up to 250 psi) their average diameter becomes progressively smaller reaching a minimum of 60-80 nm after about 5-10 passes. Moreover, as the average size is reduced, the vesicles become more and more single layered. MLVs prepared from pure PG convert to 60-70 nm single layer vesicles following about 10 passes through a 1.0 micron capillary pore membrane. The mechanism at work during such high pressure extrusion appears to be much like peeling an onion. As the MLVs are forced through the small pores, successive layers are "peeled" off until only one remains. For this method to generate truly single layered vesicles, however, the aqueous core of the starting MLV must be greater than about 70 nm in diameter. Although this appears to be the case for vesicles composed predominantly of acidic lipids, neutral vesicles or vesicles with only a few mole percent acidic lipids are not likely to convert to true single lamellar vesicles using this technique because the diameter of the inner most bilayer is probably significantly less than 70 nm. One possible exception to this rule would be neutral lipids hydrated in a nonelectrolyte solution. In this case the small amount of negative charge found as a contaminant of neutral lipids would produce enough charge repulsion to form an inner core greater than 70 nm during hydration.

Miscellaneous Methods

a. Slow Swelling in Nonelectrolyte Solutions: In 1969, Reeves and Dowben[24] reported a method for producing very large (up to several 10's of microns) single layered liposomes by allowing a thinly spread layer of hydrated phospholipids to slowly swell in distilled water or a nonelectrolyte solution. Typically, a mixture of lipids in ether or chloroform is deposited as a thin film on the bottom of a flat-bottomed beaker. The lipid is slowly hydrated by passing nitrogen gas saturated with water vapor over the film for several hours. When the film has completely hydrated it will become opaque in appearance. Following hydration, distilled water or a nonelectrolyte solution (e.g., sucrose) is carefully layered over the film and the beaker is placed in a 37°C water bath for several more hours. During this period very large single walled vesicles are formed by a mechanism which begins with single bilayers swelling and budding from the film, pinching off and eluting into the aqueous medium. The yield of single layered vesicles is good if conditions are right, but the main disadvantage of the technique is its sensitivity to any kind of mechanical agitation during vesicle formation. Also, since a very thin film is required and swelling times are long, this method would be difficult to scale up.

b. Removal of Chaotropic Ions: Oku and MacDonald[25] developed a method of forming giant single lamellar vesicles with diameters in the range of 10-20 microns by removal of sodium trichloroacetate by dialysis or dilution from a solution containing egg phospholipids and molar concentrations of sodium trichloroacetate. The yield of giant vesicles was critically dependent on the starting concentration of the chaotropic ion and temperature. Inclusion of a freeze thaw step reduced the required concentration of trichloroacetate to about 0.1 M. The giant liposomes apparently were formed from concentrations of the ion which induced the transformation of phospholipids from the lamellar phase to the micellar phase. Other chaotropic ions were also shown to be effective, including urea guanidine-HCl.

c. Freeze/Thaw: A method for the reconstitution of membrane proteins based on rapid freezing of sonicated phospholipid mixtures followed by thawing and brief sonication was originally described by Kasahara and Hinkle[26]. In 1981, Pick[27] reported that vesicles formed by this simple procedure exhibited specific trapping volumes of up to 10 µl per µmole lipid with encapsulation efficiencies of 20-30%. Formation of large liposomes by this technique probably results from the fusion of small vesicles during freezing and/or thawing of the suspension of small vesicles. This type of fusion is strongly inhibited by increasing the ionic strength of the medium, e.g., adding sucrose, and by increasing the lipid concentration. For an unexplained reason, pure phosphatidylcholine vesicles do not appear to be good candidates for this type of fusion induced growth, however. Ohu and MacDonald[28] have shown that freeze/thawing of SUVs prepared in high concentrations of alkali metal chlorides also results in the formation of giant single layered liposomes. The method involves the formation of fully hydrated small vesicles in dilute buffer by sonication followed by freeze/thawing in the presence of high concentrations of the electrolyte of interest in order to induce equilibration of the electrolyte across the bilayer membranes of the small vesicles. In the final step of the process the electrolyte concentration is reduced by dialysis against dilute buffer. This results in the influx of water into the small vesicles (driven by the osmotic imbalance) causing them to swell and fuse into giant vesicles. The method is rather involved and not easily scaled up.

d. Dehydration/Rehydration of SUVs: Large unilamellar and oligolamellar vesicles with high entrapment efficiencies have been formed by a clever method reported recently by Shew and Deamer[29]. In this method, sonicated vesicles are mixed in an aqueous solution with the solute desired to be encapsulated and the mixture dried under a stream of nitrogen. As the sample is dehydrated, the small vesicles fuse to form a multilamellar film that effectively sandwiches the solute molecules between successive layers. Upon rehydration, large vesicles are produced which have encapsulated a significant proportion of the solute. The optimal mass ratio of lipid to solute was reported to be approximately 1:2 to 1:3. This method has potential application to large scale production since it depends only on controlled drying and rehydration processes and does not require extensive use of organic solvents, detergents, or dialysis systems.

Methods for Controlling Size and Size Distribution of Liposomes

In most studies using liposomes as drug carriers, particle size has not been rigorously controlled. In studies on tissue distribution reported to date, for example, various investigations have used either the initial liposome preparation containing a wide distribution of sizes (ranging from 0.2 to 10's of microns) or sonicated vesicles which, although exhibiting a narrower size distribution, are quite small and thus have a limited capacity to carry drugs. Judging from the few studies utilizing controlled particle size, it is clear that vesicle size can have dramatic effects on the *in vivo* behavior of liposomes. Therefore, before liposome drug carrier systems can be taken seriously for pharmaceutical applications, their size will have to be controlled within reasonable limits. Three possible approaches have been explored for controlling the particle size distribution of liposome preparations: (1) fractionation of the size of interest from a heterogeneous population; (2) homogenization of a polydisperse dispersion to yield a population of smaller vesicles with a narrower size distribution; and (3) extrusion of a heterogeneous preparation through capillary pore membranes of known pore diameter to yield an average size that approximates the pore diameter.

1. Fractionation: Two methods have enjoyed widespread use for fractionating liposomes of the desired size from a heterogeneously sized population: centrifugation and size exclusion chromatography. Both can be used to enrich the product with the desired particle size but are limited in terms of the volumes that can be easily handled.

a. Centrifugation: Liposomes sediment in a centrifugal field at a rate that is dependent on their size and density. Large liposomes composed of neutral lipids such as PC can easily be pelleted at fairly low g forces in a conventional centrifuge. Under proper conditions the smaller liposomes will remain in the supernatant. This method is useful for making gross cuts between small and larger liposomes but not for generating narrow particle size distributions. Also, the volumes that can be handled are limited by the volume capacity of the centrifuge. However, zonal rotors or continuous flow centrifuges may be adaptable to this application. Another disadvantage to centrifugation is that liposomes smaller than about 0.5 micron tend to require high g forces and long spinning times in order to achieve effective separation from particles in the 0.1-0.2 micron range. Also the capacity of the ultracentrifuges normally used for this purpose is limited to a few hundred ml per run.

b. Size Exclusion Chromatography: Column chromatography has been used for many years as an analytical method to assess the particle size of liposomes. Preparative scale chromatography has also been applied to produce liposomes of fairly homogeneous sizes. This method is particularly useful for separating SUVs from larger structures. Typically, a column of Sepharose 4B is equilibrated with a buffer of the same osmolarity as the medium in which the vesicles were prepared and an aliquot of the liposomes is applied to the column. The column is eluted with the same

buffer and fractions are collected. Large liposomes appear in the void volume while SUV size liposomes elute with the included volume. Larger pore size chromatographic media have been used in a similar fashion to fractionate populations of larger particles. In general, however, such chromatographic separations are quite limited in terms of volumes and throughput, must be carried out in batches and result in significant dilution of the product.

2. Homogenization: In those cases where a fairly small particle size is desirable, homogenization has proven to be useful approach. In much the same way as milk is homogenized, the average particle size and polydispersity of vesicle dispersions can be reduced by passage through a high pressure homogenizer. One such devise marketed by the Biological Development Corporation under the trade name Microfluidizer™ has been shown by Mayhew and his colleagues[30] to generate vesicles in the 50-200 nm size range. Such homogenizers are amenable to scale up, and throughput rates are high. As with other high pressure devices, however, heat regulation can sometimes present problems, and the shear forces developed within the reaction chamber can lead to partial degradation of the lipids. Another disadvantage relates to the empirical observation that conditions designed to produce approximately 200 nm particles often results in a bimodal distribution, with the bulk of the vesicles in the desired size range contaminated by a significant proportion of very small vesicles (less than 50 nm)

3. Capillary Pore Membrane Extrusion: A technique that has gained widespread acceptance for the production of liposomes of defined size and narrow size distribution, introduced by Olson et al.[31] in 1979, involves the extrusion of a heterogeneous population of fairly large liposomes through polycarbonate membranes under moderate pressures (100-250 psi). Such membranes have uniform straight-through capillary pores of defined size and polycarbonate does not bind liposomes containing charged species. This simple technique can reduce a heterogeneous population of MLVs or REVs to a more homogeneous suspension of vesicles exhibiting a mean particle size which approaches that of the pores through which they were extruded. MLVs with a mean diameter of 260 nm can be obtained following a single extrusion through 200 nm pore size polycarbonate membranes; 75% of the encapsulated volume resides in vesicles between 170 and 370 nm (as measured by negative stain electron microscopy). Upon additional extrusions through the same pore size membrane the average size is reduced further finally approaching about 190 nm with greater than 85% of the particles in the 170-210 nm range. Compared to SUV preparations this still represents a rather broad distribution of vesicle sizes, but compared to the original MLV population which ranges in size from about 500 nm to several microns, it represents a considerable reduction of both average particle size and polydispersity. In practice it is sometimes preferable to extrude sequentially through membranes of decreasing pore diameter. For example, a concentrated dispersion of MLVs may be difficult to extrude directly through a 200

nm pore size membrane under normal operating pressures (about 90 psi). It is advisable to begin the process by extrusion through a 0.8, 0.6, 0.4 and finally 0.2 micron pore sizes. Alternatively, it is possible to use higher pressures to extrude concentrated dispersions through the smaller pore size membranes directly. A special high pressure filter holder is required, however, since operating pressures may reach 250 psi. One such devise is available commercially under the trade name LUVET™ which can accommodate up to 10 ml and is equipped with a recirculation mechanism which permits multiple extrusion with little difficulty.

Stability of Liposomes

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits for a predetermined period of time (shelf-life of the product). The first step in designing any type of stability testing program is to specify these limits by establishing parameters defined in terms of chemical stability, physical stability and microbial stability. Next, methods must be established to evaluate each of these parameters. One must treat liposomal drug delivery systems in the same way as the more traditional pharmaceutical dosage forms are treated with respect to the establishment of clearly defined protocols for their characterization, manufacture, stability testing and efficacy. General observations about liposomal stability include:

1. There are very few published reports on long-term stability studies of liposomes.
2. There are no published reports on the establishment of detailed protocols for stability testing.
3. There are no published reports on the establishment of protocols for accelerated stability testing.
4. MLVs and REVs appear to be more stable than SUVs (with respect to leakage on storage).
5. Use of saturated phospholipids and incorporation of cholesterol into the bilayer generally improves stability.
6. Liposomes stored at 4°C, at times, appear to be more stable than liposomes stored at room temperature.

Chemical Stability: Chemically, phospholipids are susceptible to hydrolysis. Additionally, phospholipids containing unsaturated fatty acids can undergo oxidative reactions. Much of the data on liposomes that have appeared in the literature can be considered suspect due to the use of phospholipids containing significant amounts of oxidation and hydrolysis products. These reaction products can cause dramatic changes in the permeability properties of liposomes. Preparative procedures (e.g., sonication) or storage conditions (e.g., exposure to different pH values) can affect the decomposition rate of the liposomal lipids.

a. <u>Lipid Peroxidation</u>: Most of the phospholipid liposomal dispersions used contain unsaturated acyl chains as part of the molecular structure. These chains are vulnerable to oxidative degradation (lipid peroxidation). The oxidation reactions can occur during preparation, storage or actual use. Oxidative deterioration of lipids is a complex process involving free radical generation and results in the formation of cyclic peroxides and hydroperoxides.

Most of the procedures used to measure lipid peroxidation are nonspecific and are either based on the disappearance of unsaturated fatty acids (determined by lipid extraction techniques followed by GLC analysis) or the appearance of conjugated dienes. The latter technique is now widely used since oxidation is accompanied by increased UV absorption in the 230-260 nm range. If unsaturated phospholipids are used to prepare liposomes, and no special precautions are used to minimize oxidation, the reaction will occur readily. Oxidation of the phospholipids may be minimized by a number of ways:

1. Minimum use of unsaturated phospholipids (if appropriate).
2. Use of argon or nitrogen to minimize exposure to O_2 .
3. Use of light resistant containers.
4. Removal of heavy metals (EDTA).
5. Use of antioxidants such as α-tocopherol or BHT.

b. <u>Lipid Hydrolysis</u>: The most important degradation product resulting from lecithin hydrolysis is lyso-lecithin (lyso-PC), which results from hydrolysis of the ester bond at the Cα position of the glycerol moiety. Many workers choose the formation of lyso-PC as a standard measure for the chemical stability of phospholipids since the presence of lyso-PC in lipid bilayers greatly enhances the permeability of liposomes. It is therefore extremely important that the formation of lyso-PC be kept to a minimum during storage. Lyso-PC is usually analyzed by phospholipid extraction followed by separation of PC and lyso-PC by TLC. The spots are then usually scraped and assayed for total phosphorous content.

Although factors such as sonication could affect the degree of lyso-PC formation, probably the single most important method of minimizing this problem is by the proper sourcing of the phospholipids to be used. They should be essentially free of any lyso-PC to start with and, of course, be free of any lipases.

c. <u>Miscellaneous Chemical Stability Concerns</u>: One must not ignore the fact that the other bilayer lipids which may be present can also decompose. For example, cholesterol, in aqueous dispersion, has been shown to oxidize rapidly when unprotected. Finally, the drug itself must be considered. The stability profile of the "free" drug may be quite different from its profile in the encapsulated state. In fact, a number of strategies have been developed which are based on protecting the drug from biological environments by encapsulating them in liposomes. Examples include the protection of insulin from proteolytic enzymes of the gastrointestinal tract and the prolongation of ester hydrolysis of prodrugs (e.g., cortisone hexadecanoate) after intramuscular administration.

Stability Testing (General Considerations)

Stability testing of liquid disperse systems is one of the most difficult problems faced by formulation chemists. The scientist is often asked to predict the shelf-life of a product or choose between experimental formulations based on estimates of how well they will hold up with time. There are no standardized tests available to determine physical stability, and quite often there is no certainty of what type of stability is being investigated. The first order of priority for solving stability problems of disperse systems is to define clearly the type or types of stability of concern. Categorizing stability as either physical or chemical is not sufficient. The various groups that are concerned with the product (product development, production, analytical, marketing, etc.) must have a clear and precise reference frame of stability.

An understanding of the factors that lead to stability problems can help determine which methods of testing are most likely to yield information applicable to the estimation of the product's shelf-life. Stability tests commonly stress the system to limits beyond those which the product will ever encounter. Typical examples of stress tests include exposure of the product to high temperatures and large gravitational forces. It is important to understand whether these tests are being performed because the product is expected to encounter these conditions or because, even though these conditions will never be approached, the results will help predict shelf-life at more moderate conditions.

High temperature testing (>25°C) is almost universally used for heterogeneous products. Various laboratories store their products at temperatures ranging from 4°C (refrigerator temperature) to 50°C (or perhaps even higher). The temperatures used in heat-cool cycling are also quite varied, often without regard for the nature of the product. What will the increase in temperature likely do to the properties of the systems under study?

For liposomes, higher temperatures may dramatically alter the nature of the interfacial film, especially if the phase transition temperature is reached. If one expects the product to be exposed to a temperature of 45°C for an extended period of time or for short durations, (shipping and warehouse storage), studies at 45-50°C, (long term and heat-cool-cycling), are quite justified. A study of a product at these temperatures determines: (1) How is the product holding up at this higher temperature, and; (2) is the damage reversible or irreversible when the product is brought back to room temperature? If temperatures higher than the system will ever encounter are used, even in short-term heat-cool cycling, there is a risk of irreversibly damaging the bilayers so that when it is brought back to room temperature, the membrane can not heal.

If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow at the expense of water. The liposomes may then be pressed together against the ice crystals under great pressure. If the crystal grows to a size greater than the void spaces, instability is more likely. That is why a slower rate of cooling, resulting in larger ice crystals, produces greater instability. Polymers may retard ice crystal growth.

van Bommel and Crommelin[32] showed that even one freeze-thaw cycle causes almost complete rapid leakage of carboxyfluorescein from liposomes (REVs) prepared from unsaturated phospholipids (even when cholesterol is added). However, liposomes composed of distearoylphosphatidylcholine/dipalmitoylphosphatidylglycerol/cholesterol show slightly better freeze-thaw stability.

Stability testing protocols should be developed for liposomal products on a case-by-case basis. A typical protocol for a product which would be shipped in vehicles not equipped with climate control and stored in warehouses for prolonged periods under similar conditions might include testing under the following conditions:

1. One month at the highest temperature likely to be encountered.
2. One month at lowest temperature likely to be encountered.
3. 12-24 months at room temperature.
4. 12-24 months at various light intensities.
5. Two to three "freeze-thaw" cycles (-20°C to 25°C).
6. Six to eight "heat-cool" cycles (5°C to 45°C, 48 hours at each temperature).
7. 24-48 hours on a reciprocating shaker at 60 cycles/min (estimates transportation conditions).

One should be certain that studies are performed using all types and sizes of containers. Under each of the test conditions, the following data can be collected:

1. Visual and microscopic observations, e.g., flocculation.
2. Particle size profiles.
3. Rheological profiles.
4. Chemical stability.
5. Extent of leakage.

Freeze Drying (Lyophilization) of Liposomes

Freeze drying involves the removal of water from products in the frozen state at extremely low pressures. The process is generally used to dry products that are thermolabile and would be destroyed by heat-drying.

Lyophilization has great potential as a method to solve long-term stability problems with respect to liposomal stability. Intuitively, one would suspect that liposomes containing drugs entrapped in their bilayers would be better candidates for lyophilization than liposomes containing drugs entrapped in their aqueous compartments since the lyophilization procedure would be expected to cause some bilayer disruption and subsequent leakage.

Various studies have shown that water-soluble markers such as carboxyfluorescein do not survive freeze drying in that even under the best of circumstances (use of saturated lipids and incorporation of cryoprotectants), a significant portion of the marker is lost on reconstitution. On the other hand, liposomes can retain >90% of lipid-soluble

drugs such as doxorubicin on reconstitution. The amount retained depends on the use of cryoprotectants, lipid composition, liposome type and loading dose.

If the leaked out drug is removed and the preparation frozen for a second time, essentially 100% of the drug is recoverable on reconstitution. This indicates that the original loss represents the portion of the drug residing in the aqueous compartment. Thus, when formulating, one must ensure that essentially all the drug is placed in the bilayer or accept a certain percentage of loss to the external medium.

Recently, it was found that trehalose, a carbohydrate commonly found at high concentrations in organisms capable of surviving dehydration, is an excellent cryoprotectant for liposomes. It may work by stabilizing the bilayers, especially at their phase transition temperatures, during both freezing and thawing[33].

STABILITY OF LIPOSOMES IN BIOLOGICAL FLUIDS

The ultimate efficacy of a liposomal dosage form will be judged on the ability of the formulator to reliably control the amount of free drug that reaches the site of action over a given period of time. Generally, the exact "site of action" or receptor site at the molecular level is not known and one relies on attaining reproducible blood levels of the drug. With traditional non-parenteral dosage forms, only the free drug is absorbed, and once the drug is in the blood stream, it has no memory about where it came from. Thus, the only method available to control the pharmacokinetics of a drug is to adjust the amount of drug that enters the blood as a function of time.

Parenteral, especially intravenous, administration of liposomally encapsulated drugs presents the formulator with additional methods to control the pharmacokinetics of the drug. Factors that affect the pharmacokinetics of parenteral liposome administration include:

a. Concentration of free drug in blood;
b. Concentration of liposomes and their entrapped drug in blood;
c. Leakage rate of drug from the liposome in the blood;
d. Disposition of the intact drug-carrying liposomes in the blood.

In order to reliably control the pharmacokinetics of these complex systems, one must be able to separate out the:

a. Stability (leakage rate) of drug from the liposome in the blood.
b. Disposition of the intact drug-carrying liposome in the blood. The pharmacokinetics of intact liposomes is beyond the scope of this review and has been thoroughly reviewed elsewhere[34].

<u>Liposome Stability in Blood and Plasma</u>: The inability of liposomes to retain entrapped substances when incubated with blood or plasma has been known for about a decade. The fact that high molecular weight substances such as inulin and even albumin leak out on incubation with plasma suggests that more than superficial damage is being done to the liposomes even though their gross morphology appears unchanged. The instability of liposomes in plasma appears to be the result of the transfer of bilayer lipids to albumin and high density lipoproteins (HDL). Additionally, some of the protein is transferred from the lipoprotein to the liposome. Both lecithin and cholesterol also exchange with the membranes of red blood cells. Liposomes are most susceptible to HDL attack at their gel to liquid crystalline phase transition temperature. It is therefore worthwhile to determine by DSC whether the formulation has a phase transition temperature close to 37°C.

The susceptibility of liposomal phospholipid to lipoprotein and phospholipase attack is strongly dependent on liposome size and type. Generally MLVs are most stable since only a portion of the phospholipid is exposed to attack and SUVs are the least stable because of the stresses imposed by their curvature. Liposomes prepared with higher chain length phospholipids are most stable both in buffer and in plasma. Incorporation of charged lipid into the bilayer decreases stability in plasma even when cholesterol is included to bring the liposomes to the gel state. Cholesterol and sphingomyelin are generally very effective in reducing the instability of liposomes in contact with plasma. It is believed that the primary reason for this effect is not the increased bilayer tightness produced by cholesterol but the prevention of transfer of phospholipid to the plasma lipoprotein and red blood cell membrane. The following table shows that liposomal stability in plasma increases as the ratio of cholesterol in the liposome increases:

TABLE 4

Release of Solutes From SUVs in the Presence of Plasma.

Liposomes	Sucrose	Inulin	PVP
PC	80.6+10.4	68.9±6.9	26.9±3.7
PC-CH (7:2)	42.2±2.8	31.1±7.2	26.1±3.0
PC-CH (7:7)	4.1±2.1	7.7±0.9	6.6

Source: Kirby, C. and Gregoriadis, G. (1981) Biochem. J., 199, 251-254.

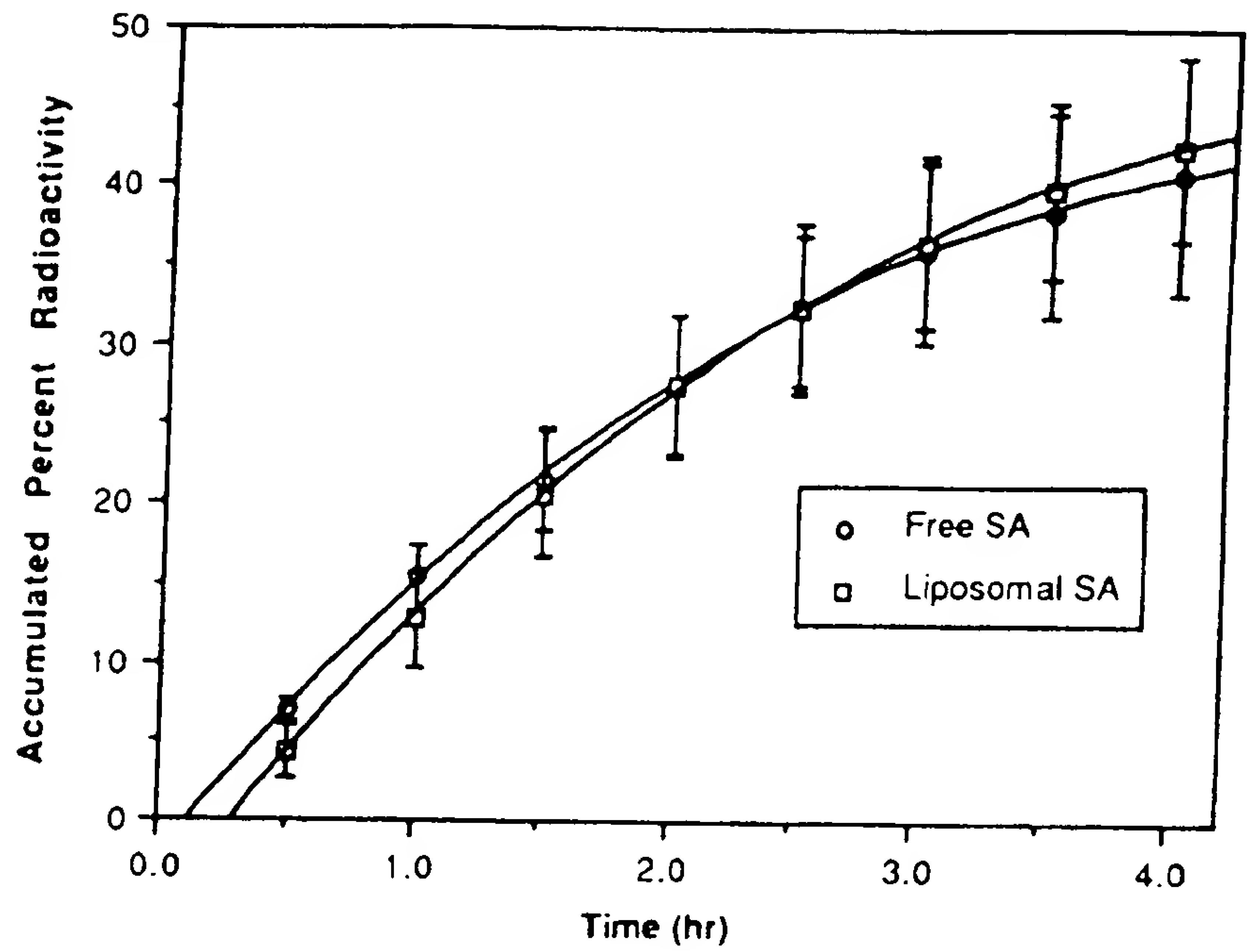

FIGURE 7
Urinary excretion profiles after oral administration of free and liposomally encapsulated (DSPC:CH; 2:1 multilamellar vesicles) salicylic acid to fasted rats. Source: Weiner, N. and Chiang, C.M. (1988) "Gastrointestinal Uptake of Liposomes" in *Liposomes as Drug Carriers*, Gregoriadis, G., Ed., p. 606.

Liposome Stability in the Gastrointestinal Tract: Although about 50 papers have been published on the oral administration of liposomally encapsulated drugs, especially insulin, very little effort has been made to critically assess stability of liposomes in the environment of the gastrointestinal tract. Rowland and Woodley[35] have shown that most of the liposomal formulations that have been used are quite unstable to the g.i. environment (low pH, bile and/or phospholipase). Even distearoylphosphatidylcholine/cholesterol liposomes are very unstable in the gastrointestinal tract and that liposomally encapsulated and free drug give about the same pharmacokinetics when administered by the oral route to rats (Fig 7).

SUMMARY

The therapeutic promise of liposomes as a drug delivery system is fast becoming a reality. One must bear in mind that only in the last five years or so have real advances made in translating progress from university laboratories into pharmaceutically acceptable dosage forms. Pharmaceutical scientists collaborating with process

engineers have been able to produce large volumes of sterile, pyrogen-free liposomes with acceptable shelf-lives. With current emphasis on increasing therapeutic indices of drugs, it appears quite likely that these biocompatible, biodegradable vehicles will receive increased attention from the pharmaceutical industry.

As of this printing, more than 10 companies plan to or have applied to the Food and Drug Administration for approval to test approximately 20 liposomally-entrapped drug entities. These drugs include anticancer and antifungal agents as well as drugs to combat arthritis, glaucoma and dry eye.

Within a short period of time one might expect to see a broad range of liposomal products in various stages of clinical testing. The most promising appear to be liposomal products specifically formulated to facilitate:

a. Site Specific Delivery: Particular emphasis is placed on disease states involving the RES. Examples include antimonial compounds for parasitic disease, immunomodulation using macrophage activating agents and antiviral treatment using ribavirin.

b. Site Avoidance Delivery: The most promising examples are liposomal doxorubicin (reduced cardiotoxicity) and liposomal amphotericin B (reduced nephrotoxicity).

c. Sustained or Controlled Release: Examples include inhalation of bronchodilators, ocular delivery of antibiotics, intramuscular delivery of peptides and topical delivery of a variety of drugs.

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EXHIBIT 6

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(54) Title: NOVEL CYANOAZIRIDINES FOR TREATING CANCER

(57) Abstract

This invention relates to novel cyanoaziridines for treatment of cancer.

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NOVEL CYANOAZIRIDINES FOR TREATING CANCER

CROSS - REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Patent Application Serial No. 60/051,037, filed June 27, 1997.

FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the invention.

This invention relates to novel cyanoaziridines for treatment of cancer.

2. Description of Related Art

This invention is directed towards aziridine-1-carboxamides related to imexon having improved anti-tumor activity.

The search for compounds having anti-tumor activity has included 2-cyanoaziridines with substituents on the nitrogen atom. German patent 2,736,296 (Feb. 22, 1979) claimed 2-cyanoaziridines and its derivatives. German patent 2,727,550 (Jan. 4, 1979) claimed 2-cyanoaziridines with substituted carbonyl, sulfonyl, or phosphoryl groups on nitrogen. East German patent 110 492 (December 20, 1974) claimed 2-cyanoaziridines with alkanoyl and aroyl substituents on nitrogen. It also claimed 2-cyanoaziridine-1-carboxamide. The cyclization of 2-cyanoaziridine-1-carboxamide to imexon, as well as imexon itself, was claimed in U.S. patent 4,083,987 (April 11, 1978). Two German patents, 2,740,248 (March 15, 1979) and 2,656,323 (June 15, 1978) claimed the preparation of

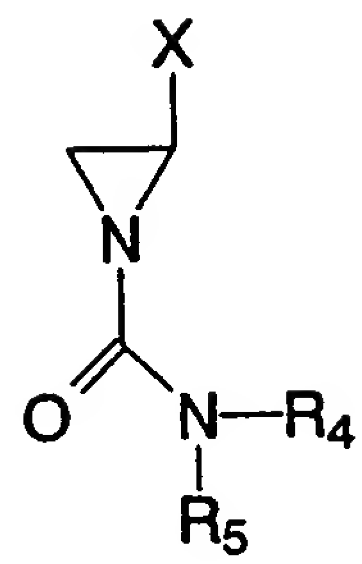
2-cyano-1-phenoxycarbonylaziridine and its conversion into 2-cyanoaziridine-1-carboxamide. Immunosuppressive activity for imidazolidine derivatives related to imexon was claimed in U.S. Patent 4,996,219.

Imexon (4-imino-1,3-diazabicyclo[3.1.0]hexan-2-one) was developed by Bicker (Immune Modulation and Control of Neoplasia by Adjuvant Therapy, M.A. Ghirigos, Ed., Raven Press, New York, 1978, p. 389) in an investigation of cyanoaziridine derivatives with potential cancerostatic action. It is a cyclic isomer of 2-cyanoaziridine-1-carboxamide, from which it is formed by treatment with a catalytic amount of KOH in methanol as illustrated below (U. Bicker, W. Kampe, and W. Steingross, U.S. Patent 4,083,987, April 11, 1978).

Imexon has both direct cytotoxicity to tumor cells and immunomodulatory effects. It is active against a variety of human tumor cell lines in culture and against fresh human tumor cell lines in clonogenic assay. It is selectively cytotoxic to multiple myeloma in the clonogenic assay (S.E. Salmon and E.M. Hersh, J. Natl. Cancer Inst., 86, 228, 1994). Imexon is active against a variety of transplanted tumors in rodents (U. Bicker and G. Hebold, IRCS Med. Sci.: Cancer; Hematology; Immunology and Allergy; Pharmacology, 5, 428, 1977; U. Bicker and P. Fuhse, Exp. Path. Bd. 10, S. 279-284, 1975) and it is active against human lymphoma, melanoma, and prostate cancer cell lines in SCID mice (Hersh, *et al.*, *Proc. Am. Assoc. Cancer Res.*, 36, 294, 1995). Objective responses were observed in dogs with mast cell tumors after treatment with imexon (R.T. Dorr, J.D. Liddil, M.K. Klein, and E.M. Hersh, Invest. New Drugs, 13, 113, 1995). Despite the presence of an aziridine ring, imexon is not myelosuppressive, which makes it potentially valuable in combination chemotherapy.

SUMMARY OF THE INVENTION

The present invention is directed to novel anti-cancer compounds of the formula:



Formula I

wherein

X is CN, CO_2R_1, or $CONR_2R_3$:

R_1 is lower alkyl, cycloalkyl, alkenyl, or aryl lower alkyl;

R_2 is hydrogen or lower alkyl;

R_3 is hydrogen lower alkyl, lower cycloalkyl, alkenyl, alkynyl, aryl, or heterocyclic ring;

R_2, R_3 and N taken together form a heterocyclic ring

R_4 is hydrogen or lower alkyl; and

R_5 is lower alkyl, lower cycloalkyl, alkenyl, alkynyl, aryl, monosubstituted aryl, disubstituted aryl, aryl lower alkyl, lower alkoxycarbonyl lower alkyl, or heterocyclic ring, with the proviso that when X is CN, and R_4 is hydrogen, then R_5 is not CH_3, C_6H_5, or, p-nitrophenyl.

R_4, R_5 and N taken together form a heterocyclic ring.

In particular, the invention is also directed to compound of the formula 1:

wherein X is CN, CO_2R_1 or $CONR_2R_3$

where R_1 is an alkyl of 1-6 carbons, a cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons or a lower alkyl substituted aryl of 7-12 carbons;

R_2 is hydrogen or lower alkyl of 1-4 carbons, and

R_3 is lower alkyl of 1-4 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons or heterocyclic ring of 4-16 ring members;

wherein R_4 is hydrogen or lower alkyl of 1-4 carbons; and,

wherein R_5 is an alkyl of 1-8 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons, a heterocyclic group of 4-16 members and where R_4, R_5 and N taken together form a heterocyclic ring of between 4 and 16 members.

The invention further includes compound of the formula 1:

wherein X is CN, CO_2R_1 or $CONR_2R_3$

where R_1 is an alkyl of 1-6 carbons, a cycloaklyl of 4-7 carbons, alkenyl of 3-6 carbons or an lower alkyl substituted aryl of 7-12 carbons;

where R_2 is hydrogen or lower alkyl of 1-4 carbons, and

where R_3 is a lower alkyl of 1-4 carbons, a lower cycloalkyl of 4-7 carbons, an alkenyl, an aryl of 4-10 carbons, a heterocyclic ring of 4-16 members or a substituted aryl or substituted heterocyclic ring where said substitutents are 1 or 2 and independently selected from the group consisting of lower alkyl of 1-4 carbons, nitro, halo substituted lower alkyls of 1-4 carbons, a lower alkyl substituted acyloxy of 1-5 carbons, a lower alkyl substituted acyl of 1-5 carbons;

wherein R_4 is hydrogen or lower alkyl of 1-4 carbons; and,

wherein R_5 is an alkyl of 1-8 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons having 1-2 substitutents wherein the substituents are independently selected from the group consisting of lower alkyl of 1-4 carbons, nitro, halo substituted lower alkyls of 1-4 carbons, a lower alkyl substituted acyloxy of 1-5 carbons, a lower alkyl substituted acyl of 1-5 carbons, a heterocyclic group of 4-16 members. Substituents R_4 and R_5 may join to form a heterocyclic ring of 4-16 members.

Preferred compounds include those wherein X is CN. Additional preferred compounds are those where X is CN and R_4 is hydrogen; and R_5 is a straight chain alkyl of 1 to 8 carbons, an unsubstituted aryl, a mono-substituted or disubstituted aryl wherein the aryl is independently substituted with halo, lower alkyl, halo substituted lower alkyl, lower alkyl-substituted acyloxy or lower alkyl-substituted acyloxy.

Also preferred are those compounds wherein X is CN and R_4 is hydrogen; and R_5 is a heterocyclic group or an unsubstituted aryl. Particularly preferred are those compounds where X is CN and R_4 is hydrogen; and R_5 is a pyridyl, a phenyl or a naphthyl.

This invention also includes the use of the above identified compounds to treat a variety of cancers by administering to a patient in need of treatment a unit dose of the compounds described above wherein said unit dose is effective to reduce at least one of the symptoms of the cancer. Preferred dose ranges are unit doses of 0.25 to 2 grams. The preferred route of administration is parental.

Specific cancers include multiple myeloma, a B-lymphocyte plasmacytoma including advanced disease refractory to alkylating agent and glucocorticosteroids, advanced stage ovarian epithelial cell cancer, including patients previously treated with alkylating agents, taxanes or platinum-containing anticancer agents, surgically unresectable (metastatic) melanoma in combination with myelosuppressive anticancer agents, multidrug-resistant leukemias of lymphoid and nonlymphoid origin including multidrug-resistant lymphomas and those lymphomas occurring in patients infected with human immunodeficiency virus-1 (AIDS), advanced stage and especially metastatic colon cancer, including those refractory to fluoropyrimidines such as 5-fluorouracil, prostate cancer, advanced stage breast cancers previously treated with alkylating agents, or natural products which induce multidrug resistance (such as doxorubicin, paclitaxel and vincristine) and metastatic lung cancers of small cell and non-small cell types which are not responsive to local radiotherapy or systemic chemotherapy with cytotoxic drugs.

In addition to novel compositions, this invention includes pharmaceutical formulations of the above identified compounds comprising a unit dose of the compounds in a sterile aqueous solution in amounts facilitating the methods of this invention requiring specific modes of administration such as intravenous administration. It is preferred that the compositions also include pharmaceutically acceptable excipients.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Median tumor volumes in SCID mice treated with AMP 404.

Figure 2. Dose response curve for imexon on 8226 myeloma cells.

Figure 3. G-banded karyotype of 8226 myeloma cells. A: 8226 sensitive cells with unidentifiable markers. B: 8226 imexon-resistant cells with unidentifiable markers.

Figure 4. Imexon induced single strand breaks in both 8226 imexon-sensitive and resistant cells (Imexon 50 mg/mL = 0.45 mM, 250 mg/mL = 2.25 mM).

DETAILED DESCRIPTION

A. INTRODUCTION.

Imexon has both direct cytotoxicity to tumor cells and immunostimulatory effects. In Phase I human clinical trials conducted in Europe in 1985, it was well tolerated and produced objective responses or stabilized neoplastic diseases in cancer patients. (Sagaster *et al*: *J Natl Cancer Inst* 87:935, 1995.)

The novel cyanoaziridines of the present invention are improvements on imexon. The novel compounds demonstrate enhanced antitumor potency (>40% decrease in IC_{50}) and there is lack of significant cross-resistance with imexon for many of the compounds. Furthermore, there are compounds roughly equal in potency to imexon, that have no significant cross-resistance in an imexon-resistant, human myeloma cell line.

The substituted derivatives of 2-cyanoaziridine have a unique mechanism of action which involves covalent binding to the sulfhydryl moieties found in a number of important cellular thiols. Studies using HPLC/Mass spectrometry have identified covalent attachment of the cyanoaziridine compounds (Molecular ion in positive mode) to the sulfur atoms on the amino acid cysteine (MH^+ = 234) and the main cellular thiol, glutathione (GSH), (MH^+ = 420). In 8226 human multiple myeloma cells. The covalent attachment occurs at the carbon of the ring-opened aziridine moiety in the cyanoaziridines. Furthermore, these cyanoaziridine drugs have been shown to deplete cysteine and GSH levels in 8226 cells direct proportion

to their ability to impair cell growth. Thus, analogs with high growth-inhibitory potency also have high potency for reducing the concentrations of both cysteine (R^2 = 0.984) and GSH (R^2 = 0.984) by Pearson product-moment correlation coefficient analyses.

As a consequence of depleting cellular thiols such as cysteine and GSH, tumor cells become highly susceptible to oxidation following exposure to cyanoaziridines. This has been documented in 8226 myeloma cells exposed to cyanoaziridines which develop high levels of peroxides such as hydrogen peroxide, and display a compensatory increase in the enzyme, GSH-peroxidase, which normally detoxifies cellular peroxides using reduced GSH. In addition, cells exposed to cyanoaziridines undergo a form of cell death known as apoptosis, or programmed cell death. This is compatible with an increase in cellular oxidants which are known to be powerful inducers of apoptosis. Human 8226 cells exposed to cyanoaziridines develop characteristic lesions of apoptosis including DNA strand breaks and also display a characteristic morphology. Tumors which rely on intracellular thiols, such as cysteine and GSH for normal growth are especially sensitive to this group of antitumor agents. This includes B-cell lymphocyte-derived tumors such as multiple myeloma as well as several type of non-hematologic (solid) tumors such as lung cancer, and malignant melanoma. Thus, the sequence of events for cell killing with the cyanoaziridines involves (1) depletion of thiols by binding to critical sulfur atoms in amino acids, peptides and proteins, (2) a buildup of organic oxidants including peroxides, and (3) induction of the apoptotic form of cell death.

There are no existing anticancer agents which deplete cellular thiols. Furthermore, there are relatively few agents which are non-myelosuppressive (do not damage the bone marrow) as has been demonstrated for the cyanoaziridine-based agents.

B. DEFINITIONS.

As used herein, the term "alkyl" when used alone or in combination, consists of a carbon chain containing from one to eight carbon atoms. The alkyl

groups may be a straight chain or a branched chain. It includes such groups as methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, t-butyl, n-pentyl, amyl, n-hexyl, and the like. The preferred alkyl groups are methyl and ethyl. Lower alkyls are C1-4 and higher alkyls are C_5-C_8.

"Substituted alkyl" refers to alkyl as just described including one or more functional groups such as lower alkyl, aryl, acyl, halogen (i.e., alkylhalos, *e.g.*, CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, aryloxy, aryloxyalkyl, mercapto, both saturated and unsaturated cyclic hydrocarbons, heterocycles and the like. These groups may be attached to any carbon of the alkyl moiety.

The term "aryl" is used herein to refer to an aromatic substituent which may be a single aromatic ring or multiple aromatic rings which are fused together, linked covalently, or linked to a common group such as a methylene or ethylene moiety. The common linking group may also be a carbonyl as in benzophenone. The aromatic ring(s) may include phenyl, napthyl, biphenyl, diphenylmethyl and benzophenone among others. The term "aryl" encompasses "arylalkyl."

The term "arylalkyl" is used herein to refer to a subset of "aryl" in which the aryl group is attached to the nucleus shown in Formula 1 by an alkyl group as defined herein.

"Substituted aryl" refers to aryl as just described including one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (*e.g.*, CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, mercapto and both saturated and unsaturated cyclic hydrocarbons which are fused to the aromatic ring(s), linked covalently or linked to a common group such as a methylene or ethylene moiety. The linking group may also be a carbonyl such as in cyclohexyl phenyl ketone. The term "substituted aryl" encompasses "substituted arylalkyl."

"Substituted arylalkyl" defines a subset of "substituted aryl" wherein the substituted aryl group is attached to the nucleus shown in Formula 1 by an alkyl group as defined herein.

The term "acyl" is used to describe a ketone substituent, —C(O)R, where R is alkyl or substituted alkyl, aryl or substituted aryl as defined herein.

The term "halogen" is used herein to refer to fluorine, bromine, chlorine and iodine atoms.

The term "hydroxy" is used herein to refer to the group —OH.

The term "amino" is used to describe primary amines, R—NH_2.

The term "alkoxy" is used herein to refer to the —OR group, where R is a lower alkyl, substituted lower alkyl, aryl, substituted aryl, arylalkyl or substituted arylalkyl wherein the alkyl, aryl, substituted aryl, arylalkyl and substituted arylalkyl groups are as described herein. Suitable alkoxy radicals include, for example, methoxy, ethoxy, phenoxy, substituted phenoxy, benzyloxy, phenethyloxy, t-butoxy, *etc*.

The term "alkylamino" denotes secondary and tertiary amines wherein the alkyl groups may be either the same or different and may consist of straight or branched, saturated or unsaturated hydrocarbons.

As used herein, the term "acylamino" describes substituents of the general formula RC(O)NR', wherein R' is a lower alkyl group and R represents the nucleus shown in Formula 1 or an alkyl group, as defined herein, attached to the nucleus.

The term "acyloxy" is used herein to describe an organic radical derived from an organic acid by the removal of the acidic hydrogen. Simple acyloxy groups include, for example, acetoxy, and higher homologues derived from carboxylic acids such as ethanoic, propanoic, butanoic, *etc*. The acyloxy moiety may be oriented as either a forward or reverse ester (i.e., RC(O)OR' or R'OC(O)R, respectively, wherein R comprises the portion of the ester attached either directly or through an intermediate hydrocarbon chain to the nucleus shown in claim 1).

As used herein, the term "aryloxy" denotes aromatic groups which are linked to the nucleus shown in Formula 1 directly through an oxygen atom. This term encompasses "substituted aryloxy" moieties in which the aromatic group is substituted as described above for "substituted aryl."

As used herein "aryloxyalkyl" defines aromatic groups attached, through an oxygen atom to an alkyl group, as defined herein. The alkyl group is attached to

the nucleus shown in Formula 1. The term "aryloxyalkyl" encompasses "substituted aryloxyalkyl" moieties in which the aromatic group is substituted as described for "substituted aryl."

As used herein, the term "mercapto" defines moieties of the general structure R—S—R' wherein R and R' are the same or different and are alkyl, aryl or heterocyclic as described herein.

The term "saturated cyclic hydrocarbon" denotes groups such as the cyclopropyl, cyclobutyl, cyclopentyl, *etc.*, and substituted analogues of these structures.

The term "unsaturated cyclic hydrocarbon" is used to describe a monovalent non-aromatic group with at least one double bond, such as cyclopentene, cyclohexene, *etc.* and substituted analogues thereof.

The term "heteroaryl" as used herein refers to aromatic rings in which one or more carbon atoms of the aromatic ring(s) are substituted by a heteroatom such as nitrogen, oxygen or sulfur. Heteroaryl refers to structures which may be a single aromatic ring, multiple aromatic ring(s), or one or more aromatic rings coupled to one or more non-aromatic ring(s). In structures having multiple rings, the rings can be fused together, linked covalently, or linked to a common group such as a methylene or ethylene moiety. The common linking group may also be a carbonyl as in phenyl pyridyl ketone. As used herein, rings such as thiophene, pyridine, isoxazole, phthalimide, pyrazole, indole, furan, *etc.* or benzo-fused analogues of these rings are defined by the term "heteroaryl."

"Heteroarylalkyl" defines a subset of "heteroaryl" wherein an alkyl group, as defined herein, links the heteroaryl group to the nucleus shown in Formula 1.

"Substituted heteroaryl" refers to heteroaryl as just described wherein the heteroaryl nucleus is substituted with one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (*e.g.*, CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, mercapto, *etc.* Thus, substituted analogues of heteroaromatic rings such as thiophene, pyridine, isoxazole, phthalimide, pyrazole, indole, furan, *etc.*

or benzo-fused analogues of these rings are defined by the term "substituted heteroaryl."

"Substituted heteroarylalkyl" refers to a subset of "substituted heteroaryl" as described above in which an alkyl group, as defined herein, links the heteroaryl group to the nucleus shown in Formula 1.

The term "heterocyclic" is used herein to describe a monovalent saturated or unsaturated non-aromatic group having a single ring or multiple condensed rings from 1-12 carbon atoms and from 1-4 heteroatoms selected from nitrogen, sulfur or oxygen within the ring. Such heterocycles are, for example, tetrahydrofuran, morpholine, piperidine, pyrrolidine, *etc.*

The term "substituted heterocyclic" as used herein describes a subset of "heterocyclic" wherein the heterocycle nucleus is substituted with one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (*e.g.*, CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, mercapto, *etc.* It is preferred that the heterocyclic ring contain 5 or 6 ring atoms.

The term "heterocyclicalkyl" defines a subset of "heterocyclic" wherein an alkyl group, as defined herein, links the heterocyclic group to the nucleus shown in Formula 1.

The term "substituted heterocyclicalkyl" defines a subset of "heterocyclic alkyl" wherein the heterocyclic nucleus is substituted with one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (*e.g.*, CF_3), hydroxy, amino, alkoxy, alkylamino, acylamino, acyloxy, mercapto, *etc.*

"Alkylene" refers herein to a divalent lower alkyl substituent as defined above, such as methylene ($-CH_2-$), ethylene ($-CH_2CH_2-$) or propylene ($-CH_2CH_2CH_2-$). "Substituted alkylene" refers to alkylene as just described including one or more functional groups such as lower alkyl, aryl, aralkyl, acyl, halogen, hydroxyl, amino, acylamino, acyloxy, alkoxyl, mercapto and the like.

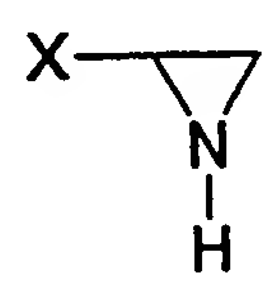
"Alkenylene" refers herein to a divalent lower alkyl substituent having one or more double bonds, such as ethenylene ($-CH=CH-$). "Alkynylene" refers herein to a divalent lower alkyl substituent having one or more triple bonds, such as

ethynylene (-C≡C-). "Substituted alkenylene" and "substituted alkynylene" refer to an alkenylene or an alkynylene as just described including one or more functional groups such as lower alkyl, aryl, aralkyl, acyl, halogen, hydroxyl, amino, acylamino, acyloxy, alkoxyl, mercapto and the like.

Unless otherwise stated: (i) all numerical ranges are inclusive, *i.e.*, 1-3 or 1 to 3 carbons includes 1,2 and 3 carbons; (ii) heterocyclical substituents may be attached through any available hydrogen that would exist in the non-radical form of the heterocycle member.

C. SYNTHESIZING IMEXON RELATED CYANOAZIRIDINES.

The basic nucleus of Formula II, wherein X is CN can be made in accordance with the method of Jänisch, *et al.* **(Jänisch, *et al., Synthesis* 1992, 1211-1212, 1992.)** Briefly, 2,3-dibromopropionitrile is treated at 5-15 °C with ammonia and then triethanolamine is added and the mixture is heated at reflux temperature. Following workup, the product is distilled under reduced pressure.



Formula II

When X is CO_2R_1, the basic nucleus is made by the method of Kyburz, *et al.* (Kyburz, *et al., Helv Chim Acta* 49:359-369, 1968.) In this method, esters of 2,3-dibromopropionic acid are stirred with N-phenyl-2-naphthylamine. The ammonia is then evaporated and the product is worked up and distilled under reduced pressure. A variant of this method uses the corresponding esters of 2-bromoacrylic acid in place of the esters of 2,3-dibromopropionic acid. (Kyburz, *et al., Helv Chim Acta* 49:359-369, 1968.)

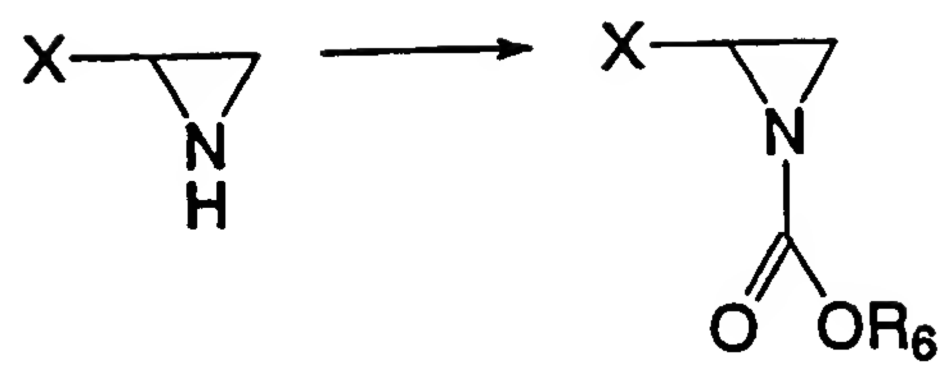
Compounds containing the basic nucleus wherein X is $CONR_2R_3$ are made by treating methyl or ethyl aziridine-2-carboxylate, prepared as described above, with ammonia or appropriate amines in methanol solution. In the case where R_2 and R_3 are H, evaporation of solvent gave the product quantitatively according to

Kyburz, *et al.* (Kyburz, *et al., Helv Chim Acta* 49:359-369, 1968). If purification is necessary,the products may be distilled at low pressure (1-10 mm) or recrystallized. Many appropriate amines are commercially available. They include, but are not limited to alkyl (methylamine, *etc.*), dialkyl (diethylamine, *etc.*), alkenyl (allylamine), alkynyl (propargylamine), aryl (aniline, *etc.*), and heterocyclic (pyrrolidine, *etc.*).

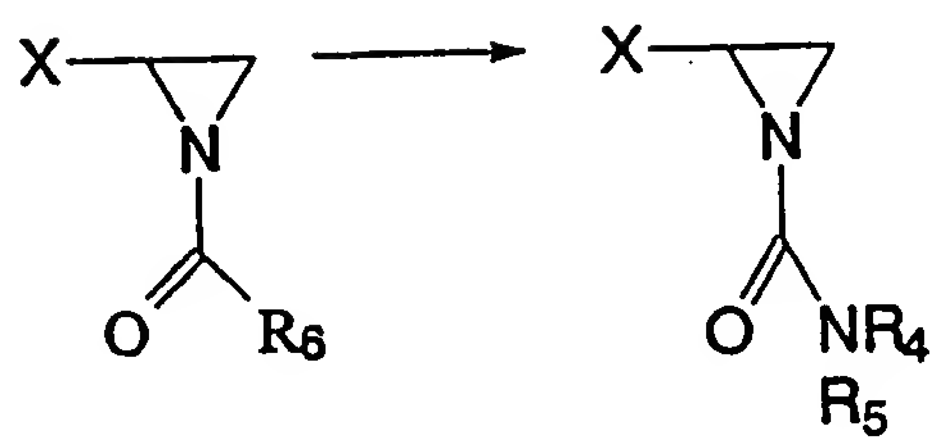
Compounds possessing the basic nucleus can be converted into the compounds of this invention by two different methods. The preferred method depends on the structure of the product and the availability of appropriate isocyanate reagents.

When R_4 is H and isocyanates are commercially available or easily prepared, the preferred method is treatment of the basic nucleus with an isocyanate in an inert solvent such as benzene or toluene until complete disappearance of the starting material is indicated by thin-layer chromatography. Generally the product crystallizes when the reaction mixture is cooled. If not, the solvent is removed under reduced pressure to provide the product. Many appropriate isocyanates are commercially available. They include, but are not limited to alkyl (methylisocyanate, *etc.*), lower cycloalkyl (cyclohexylisocyanate), alkenyl (allylisocyanate), aryl (phenylisocyanate), monosubstituted aryl (tolylisocyanate, *etc.*), disubstituted aryl (3,4-dichlorophenylisocyanate), aryl lower alkyl (benzylisocyanate), and lower alkoxycarbonyl lower alkyl ($CH_2CO_2C_2H_5$). In other cases, the isocyanate can be prepared from an available intermediate. For example, 3-pyridylisocyanate is made by heating nicotinic acid azide in toluene by the procedure of Hyden and Wilbert. (Hyden, *et al.*, *Chem Ind* (London) 3:1406-1407, 1967.)

When R_4 is not H or when an appropriate isocyanate is not available, the preferred method for preparing the compounds of this invention is to first convert the basic nucleus into a carbamate by treating it with 1 to 1.2 equivalents of a chloroformate such as 4-nitrophenyl chloroformate or trichloromethyl chloroformate (reaction 1) in an inert solvent such as benzene, chloroform, or tetrahydrofuran at low temperature (5-20°C) and removing the solvent under reduced pressure.



R_6 is $C_6H_4N_4NO_2$ or CCl_3



The resulting carbamate is treated with appropriate primary or secondary amines to give the desired product (reaction 2). Conditions for conducting this reaction are to treat the carbamate with 1 to 1.2 equivalents of the amine in an inert solvent such as toluene, chloroform, or tetrahydrofuran at room temperature until thin-layer chromatography indicates complete consumption of the carbamate. Many appropriate amines are commercially available. They include, but are not limited to dialkyl (diethylamine, *etc.*), alkynyl (propargylamine), monosubstituted aryl (2-napthylamine), disubstituted aryl (4-aminobenzoic), aryl lower alkyl (phenethylamine), heterocyclic (piperidine, *ect.*), and heteroaryl (2-aminothiazole).

The compounds of this invention can be purified by recrystallization from appropriate chemically inert solvents such as toluene, chloroform, and ethyl acetate.

D. TESTING NOVEL IMEXON-RELATED CYANOAZIRIDINES FOR ANTICANCER ACTIVITY.

The compounds described above have anticancer activity. A number of biological assays are available to evaluate and to optimize the choice of specific compounds for optimal antitumor activity. These assays can be roughly split into two

groups those involving *in vitro* exposure of agents to tumor cells and *in vivo* antitumor assays in rodent models and rarely, in larger animals.

The *in vitro* experiments for new anticancer agents generally involve the use of established tumor cell lines both of animal and, especially of human origin. These cell lines can be obtained from commercial sources such as the American Type Tissue Culture Laboratory in Bethesda, Maryland and from tumor banks at research institutions. Exposures to new agents are carried out under simulated physiological conditions of temperature, oxygen and nutrient availability in the laboratory. The endpoints for these *in vitro* assays can involve: 1) colony formation; 2) a simple quantitation of cell division over time; 3) the uptake of so called "vital" dyes which are excluded from cells with an intact cytoplasmic membrane; or 4) the incorporation of radiolabeled nutrients into a proliferating (viable) cell. Colony forming assays have been used both with established cell lines, as well as fresh tumor biopsies surgically removed from patients with cancer. In this type of assay, cells are typically grown in petri dishes on soft agar, and the number of colonies or groups of cells (> 60 μ in size) are counted either visually, or with an automated image analysis system. A comparison is then made to the untreated control cells allowed to develop colonies under identical conditions. Because colony formation is one of the hallmarks of the cancer phenotype only malignant cells will form colonies without adherence to a solid matrix. This can therefore be used as a screening procedure for new agents, and there are a number of publications which show that results obtained in colony forming assays correlates with clinical trial findings with the same drugs.

The enumeration of the total number of cells is a simplistic approach to *in vitro* testing with either cell lines or fresh tumor biopsies. In this assay, clumps of cells are typically disaggregated into single units which can then be counted either manually on a microscopic grid or using an automated flow system such as either flow cytometry or a Coulter® counter. Control (untreated) cell growth rates are then compared to the treated cell growth rates. Vital dye staining is another one of the older hallmarks of antitumor assays. In this type of approach cells, either untreated

or treated with a cancer drug, are subsequently exposed to a dye such as methylene blue, which is normally excluded from intact (viable) cells. The number of cells taking up the dye (dead or dying) are the numerator with a denominator being the number of cells which exclude the dye. These are laborious assays which are not currently used extensively due to the time and the relatively non-specific nature of the endpoint.

In addition to vital dye staining, viability can be assessed using the incorporation of radiolabeled nutrients and/or nucleotides. This is the test method that was used in the Viking Lander to look for life on Mars with the endpoint being how much of a radioactive substance was taken up into a sample as evidence of life activity. In tumor cell assays, a typical experiment involves the incorporation of either (^{3}H) tritium or ^{14}C-labeled nucleotides such as thymidine. Control (untreated) cells are shown to take up a substantial amount of this normal DNA building block per unit time, and the rate of incorporation is compared to that in the drug treated cells. This is a rapid and easily quantitatable assay that has the additional advantage of working well for cells that may not form large (countable) colonies. Drawbacks include the use of radioisotopes which present handling and disposal concerns.

There are large banks of human and rodent tumor cell lines that are available for these types of assays. The current test system used by the National Cancer Institute uses a bank of over 60 established sensitive and multidrug -resistant human cells lines of a variety of cell subtypes. This typically involves 5-6 established and well-characterized human tumor cells of a particular subtype, such as non-small cell or small cell lung cancer, for testing new agents. Using a graphic analysis system called Compare®, the overall sensitivity in terms of dye uptake (either sulforhodamine B or MTT tetrazolium dye) are utilized. The specific goal of this approach is to identify compounds that are uniquely active in a single histologic subtype of human cancer. In addition, there are a few sublines of human cancer that demonstrate resistance to multiple agents and are known to, in some cases, express the multidrug resistance pump, p-glycoprotein. Assays using these resistant cells

are currently underway for screening compounds both from NCI laboratories as well as any submitted from universities or private parties. The endpoint for the NCI assay is the incorporation of a protein dye called sulforhodamine B (for adherent tumor cells) and the reduction of a tetrazolium (blue) dye in active mitochondrial enzymes (for non-adherent, freely-floating types of cells). This latter method is particularly useful for hematologic cancers including myelomas, leukemias and lymphomas.

Generally, once compounds have demonstrated some degree of activity *in vitro* at inhibiting tumor cell growth, such as colony formation or dye uptake, antitumor efficacy experiments are performed *in vivo*. Rodent systems are almost exclusively used for initial assays of antitumor activity since tumor growth rates and survival endpoints are well-defined, and since these animals generally reflect the same types of toxicity and drug metabolism patterns as in humans. For this work, syngeneic (same gene line) tumors are typically harvested from donor animals, disaggregated, counted and then injected back into syngeneic (same strain) host mice. Cancer drugs are typically then injected at some later time point(s), either by intraperitoneal, intravenous or oral routes, and tumor growth rates and/or survival are determined, compared to untreated controls. In these assays, growth rates are typically measured for tumors injected growing in the front flank of the animal, wherein perpendicular diameters of tumor width are translated into an estimate of total tumor mass or volume. The time to reach a predetermined mass is then compared to the time required for equal tumor growth in the untreated control animals. Significant findings generally involve a > 25% increase in the time to reach the predetermined mass in the treated animals compared to the controls. This is termed tumor growth inhibition. For non-localized tumors such as leukemia, survival can be used as an endpoint and a comparison is made between the treated animals and the untreated or solvent treated controls. In general, a significant increase in life span for a positive new agent is again > 20-25% longer life span due to the treatment. Early deaths, those occurring before any of the untreated controls, generally indicate toxicity for a new compound.

For all these assays, the cancer drugs are generally tested at doses very near the lethal dose and 10% (LD_{10}) and/or at the determined maximally-tolerated dose, that dose which produces significant toxicity, but no lethality in the same strain of animals and using the same route of administration and schedule of dosing. Similar studies can also be performed in rat tumor models although, because of the larger weight and difficulty handling these animals they are less preferred than the murine models.

More recently, human tumors have been successfully transplanted in a variety of immunologically deficient mouse models. In the initial work, a mouse called the nu/nu or "nude" mouse was used to develop *in vivo* assays of human tumor growth. In nude mice, which are typically hairless and lack a functional thymus gland, human tumors (millions of cells) are typically injected in the flank and tumor growth occurs slowly thereafter. This visible development of a palpable tumor mass is called a "take". Anticancer drugs are then injected by some route (IV, IM, SQ, PO) distal to the tumor implant site, and growth rates are calculated by perpendicular measures of the widest tumor widths as described earlier. A number of human tumors are known to successfully "take" in the nude mouse model, even though these animals are more susceptible to intercurrent infections due to the underlying immunologic deficiency. An alternative mouse model for this work involves mice with a severe combined immunodeficiency disease (SCID) wherein there is a defect in maturation of lymphocytes. Because of this, SCID mice do not produce functional B- and T-lymphocytes. However, these animals do have normal cytotoxic T-killer cell activity. Nonetheless, SCID mice will "take" a large number of human tumors. Animals with the SCID phenotype are screened for "leakiness" by measuring serum immunoglobulin production which should be minimal to undetectable if the SCID phenotype is maintained. Tumor measurements and drug dosing are generally performed as above. The use of SCID mice has in many cases displaced the nude mouse since SCID mice seem to have a greater ability to take a larger number of human tumors and are more robust in terms of lack of sensitivity to

intercurrent infections. Again, positive compounds in the SCID mouse model are those that inhibit tumor growth rate by > 20-25% compared to the untreated control.

Testing for drug resistance can involve any of the *in vitro* and *in vivo* models, although the *in vitro* models are better characterized. In these tests, a cell subline is developed for resistance to a particular agent generally by serial exposure to increasing concentrations of the drug either *in vitro* or rarely *in vivo*. Once a high degree of resistance is demonstrated (generally > 4- to 5-fold) to a particular agent the cell line is further studied for mechanisms of resistance such as the expression of multidrug resistance membrane pumps such as p-glycoprotein or others. These resistant cell lines can then be tested for cross-resistance with other classic anticancer agents to develop a response pattern for a particular cell line. Using this cell line one can then evaluate a new agent for its potential to be active in the resistant cells. This has allowed for the demonstration of both mechanisms of drug resistance, as well as the identification of agents which might have utility in human cancers that have become resistant to existing chemotherapy agents. More recently, the use of resistant human tumor cells has been extended to the SCID mouse model with the development of an *in vivo* model of multidrug-resistant human multiple myeloma.

All of these test systems are generally combined in a serial order, moving from *in vitro* to *in vivo*, to characterize the antitumor activity of a new agent. In general, one wishes to find out what tumor types are particularly sensitive to a new drug and conversely what tumor types are intrinsically resistant to a new agent *in vitro*. Using this information, experiments are then planned in rodent models to evaluate whether or not the compounds that have shown activity *in vitro* will be tolerated and active in animals. The initial experiments in animals generally involve toxicity testing to determine a tolerable dose schedule and then using that dose schedule, to evaluate antitumor efficacy as described above. Active compounds from these two types of assays may then be tested in human tumors growing in SCID or nude mice and if activity is confirmed, these drugs then become candidates for potential clinical drug development.

E. FORMULATING IMEXON-RELATED CYANOAZIRIDINES.

Various compositions of the present invention are presented for administration to humans and animals in unit dosage forms, such as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, eye drops, oral solutions or suspensions, and water-in-oil emulsions containing suitable quantities of compounds of Formula I.

The term "unit dosage form", as used in the specification, refers to physically discrete units suitable as unitary dosages for human subjects and animals, each unit containing a predetermined quantity of active material calculated to produce the desired pharmaceutical effect in association with the required pharmaceutical diluent, carrier or vehicle. The specifications for the novel unit dosage forms of this invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for use in humans and animals, as disclosed in detail in this specification, these being features of the present invention. Examples of suitable unit dosage forms in accord with this invention are tablets, capsules, pills, suppositories, powder packets, wafers, granules, cachets, teaspoonfuls, tablespoonfuls, dropperfuls, ampoules, vials, aerosols with metered discharges, segregated multiples of any of the foregoing, and other forms as herein described.

An effective quantity of the drug is employed in treatment. The dosage of the specific compound for treatment depends on many factors that are well known to those skilled in the art. They include for example, the route of administration and the potency of the particular compound. A dosage schedule for humans of from about 0.25 to 2 grams of compound in a single dose, administered parenterally or in the compositions of this invention, are effective for treating different types of cancer.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compounds and a sterile vehicle, 0.9% sodium chloride being preferred. The cyanoaziridine-derivative, depending on the vehicle and concentration used, can be either suspended or dissolved in the vehicle. In preparing solutions the

compound can be dissolved in water for injection and filtered sterilized before filling into a suitable vial or ampule and sealing. Advantageously, adjuvants such as a local anesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection is supplied to reconstitute the liquid prior to use. Parenteral suspensions can be prepared in substantially the same manner except that the compounds are suspended in the vehicle instead of being dissolved and sterilization cannot be accomplished by filtration. The compound can be sterilized by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the cyanoaziridine derivative.

For oral administration, either solid or fluid unit dosage forms can be prepared. For preparing solid compositions such as tablets, the compounds of formula I are mixed with conventional ingredients such as talc, magnesium stearate, dicalcium phosphate, magnesium aluminum silicate, calcium sulfate, starch, lactose, acacia, methylcellulose, and functionally similar materials as pharmaceutical diluents or carriers. Capsules are prepared by mixing the compound with an inert pharmaceutical diluent and filling the mixture into a hard gelatin capsule of appropriate size. Soft gelatin capsules are prepared by machine encapsulation of a slurry of the compound with an acceptable vegetable oil, light liquid petrolatum or other inert oil.

Fluid unit dosage forms for oral administration such as syrups, elixirs, and suspensions can be prepared. The water-soluble forms can be dissolved in an aqueous vehicle together with sugar, aromatic flavoring agents and preservatives to form a syrup. An elixir is prepared by using a hydroalcoholic (ethanol) vehicle with suitable sweeteners such as sugar and saccharin, together with an aromatic flavoring agent.

Suspensions can be prepared with an aqueous vehicle with the aid of a suspending agent such as acacia, tragacanth, methylcellulose and the like.

Surfactants such as Cremophor EL (polyethoxylated castor oil) or polysorbate 80 (Tween®-80) may also be included.

Additionally, a suppository can be employed to deliver the drug. The active compound can be incorporated into any of the known suppository bases by methods known in the art. Examples of such bases include cocoa butter, polyethylene glycols (carbowaxes), polyethylene sorbitan monostearate, and mixtures of these with other compatible materials to modify the melting point or dissolution rate. These suppositories can weigh from about 1 to 2.5 grams.

Implants comprising polymeric devices which slowly release or slowly erode and release within the body to provide continuous supplies of the cyanoaziridine derivative are also of use. Implants include subcutaneous devices such as those routinely used to deliver the hormonal antitumor agent leuprolide or goserelin and other medicaments. Other implants include intratumoral and intraarterial devices.

The implants may be made of polymers which generally comprise but are not limited to non-toxic hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers and other biodegradable polymers. Hydrogels include polyhydroxyalkyl methacrylates, polyacrylamide and polymethacrylamide, polyvinylpyrrolidone and polyvinyl alcohol. A preferred silicone is polydimethylsiloxane. Biodegradable polymers include polylactic acid [PLA], polyglycolic acid [PGA], copolymers of PLA and PGA, and polyamides.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, practice the present invention to its fullest extent. The following detailed examples describe how to prepare the various compounds and/or perform the various processes of the invention and are to be construed as merely illustrative, and not limitations of the preceding disclosure in any way whatsoever. Those skilled in the art will promptly recognize appropriate variations from the procedures both as to reactants and as to reaction conditions and techniques.

F. TREATMENT OF HUMAN CANCERS WITH NOVEL CYANOAZIRIDINES.

In general, anticancer therapy is given cyclicly on an every 3 or 4 week basis in order to reduce the tumor load and allow for recovery of normal cells from toxicity. The cyanoaziridine derivative drugs are given by an oral or parenteral route either as a single injection of a large dose or as a series of small daily doses for up to a 4 week period of continuous daily dosing. The continuous dosing regimen may be performed either as a series repeated daily injections, the injection of one large slow release depot formulation (in a subcutaneous or intramuscular site), or the intravenous or intraarterial infusion of agent continuously for several sequential days. Dosing regimens are determined to maximize activity with acceptable, but moderate to severe toxicities. Because the parent cyanoaziridine derivative imexon has not demonstrated myelosuppressive toxicity, it may be possible to continue dosing for prolonged periods with this agent in order to significantly reduce tumor burden and extend survival. These daily injections are given either as a rapid I.V. administration or as a continuous infusion or as an implantation of a slow release formulation in a subcutaneous or intramuscular site.

Measuring Response to Cyanoaziridines

Tumor load is assessed prior to therapy by means of objective scans of the tumor such as with x-ray radiographs, computerized tomography (CAT scans), nuclear magnetic resonance (NMR) scans or direct physical palpation of the tumor mass. Alternatively, the tumor may secrete a marker substance such as alphafetoprotein from colon cancer, CA125 antigen from ovarian cancer, or serum myeloma "M" protein from multiple myeloma. The levels of these secreted products then allow for an estimate of tumor burden to be calculated. These direct and indirect measures of the tumor load are done pretherapy, and are then repeated at intervals following the administration of the drug in order to gauge whether or not an objective response has been obtained. An objective response in cancer therapy generally indicates > 50% shrinkage of the measurable tumor disease (a partial response), or complete disappearance of all measurable disease (a complete

response). Typically these responses must be maintained for a certain time period, usually one month, to be classified as a true partial or complete response. In addition, there may be stabilization of the rapid growth of a tumor or there may be tumor shrinkage that is < 50%, termed a minor response. In general, increased survival is associated with obtaining a complete response to therapy and in some cases, a partial response if maintained for prolonged periods can also contribute to enhanced survival in the patient. Patients receiving chemotherapy are also typically "staged" as to the extent of their disease before and following chemotherapy are then restaged to see if this disease extent has changed. In some situations the tumor may shrink sufficiently and if no metastases are present, then surgical excision may be possible after chemotherapy treatment where it was not possible beforehand due to the widespread disease. In this case the chemotherapy treatment with the novel cyanoaziridine is being used as an adjuvant to potentially curative surgery. In addition, patients may have individual lesions in the spine or elsewhere that produce symptomatic problems such as pain and these may need to have local radiotherapy applied. This may be done in addition to the continued use of the systemic cyanoaziridine.

Assessing Cyanoaziridine Toxicity and Setting Dosing Regimens

Patients are assessed for toxicity with each course of chemotherapy, typically looking at effects on liver function enzymes and renal function enzymes such as creatinine clearance or BUN as well as effects on the bone marrow, typically a suppression of granulocytes important for fighting infection and/or a suppression of platelets important for hemostasis or stopping blood flow. For such myelosuppressive drugs, the nadir in these normal blood counts, is reached between 1-3 weeks after therapy and recovery then ensues over the next week. Based on the recovery of normal white blood counts, treatments may then be resumed. However, because the cyanoarizidines have not demonstrated serious myelosuppression (bone marrow) toxicity to date, they may be used more frequently in a process called "dose intensification." This indicates the more frequent dosing of

a cyanoaziridine agent as a means of achieving more substantial reduction in tumor burden.

In general, complete and partial responses are associated with at least a 1-2 log reduction in the number of tumor cells (a 90-99% effective therapy). Patients with advanced cancer will typically have $>10^9$ tumor cells at diagnosis, multiple treatments will be required in order to reduce tumor burden to a very low state and potentially obtain a cure of the disease.

Using Cyanoaziridines in Combination With Other Agents

Because of the lack of myelotoxicity, the cyanoaziridines may be combined with other existing cytotoxic agents including other drugs which damage the bone marrow. In this regard the cyanoaziridines may offer a distinct advantage over existing agents because they could be combined at full dose with the full dose of bone marrow suppressing drugs due to the lack of myelosuppression for the cyanoaziridines. This has been a major advantage with the use of the nonmyelosuppressive vinca alkaloid, vincristine, in patients with acute leukemia. Similarly, the cyanoaziridines may be combined with a large number of existing myelosuppressive agents in order to provide high dose intensity with multiple agent therapy.

The use of multiple chemotherapy agents is desirable in order to produce a major reduction in tumor load as well as to prevent the emergence of cells resistant to a single therapy.

Clinical Management of Patients Receiving Cyanoaziridines

At the end of a treatment cycle with the cyanoaziridine derivative which could comprise several weeks of continuous drug dosing, patients will be evaluated for response to therapy (complete and partial remissions), toxicity measured by blood work and general well-being classified performance status or quality of life analysis. The latter includes the general activity level of the patient and their ability to do normal daily functions. It has been found to be a strong predictor of response and

some anticancer drugs may actually improve performance status and a general sense of well-being without causing a significant tumor shrinkage. The antimetabolite gemcitabine is an example of such a drug that was approved in pancreatic cancer for benefiting quality of life without changing overall survival or producing a high objective response rate. Thus, for some cancers that are not curable, the cyanoaziridines may similarly provide a significant benefit, well-being performance status, etc. without affecting true complete or partial remission of the disease.

In hematologic disorders such as multiple myeloma, lymphoma and leukemia, responses are not assessed via the measurement of tumor diameter since these diseases are widely metastatic throughout the lymphatic and hematogenous areas of the body. Thus, responses to these diffusely disseminated diseases are usually measured in terms of bone marrow biopsy results wherein the number of abnormal tumor cell blasts are quantitated and complete responses are indicated by the lack of detection of any tumor cells in a bone marrow biopsy specimen. With the B-cell neoplasm multiple myeloma a serum marker, the M protein, can be measured by electrophoresis and if substantially decreased this is evidence of the response of the primary tumor. Again, in multiple myeloma, bone marrow biopsies can be used to quantitate the number of abnormal tumor plasma cells present in the specimen. For these diseases generally higher dose therapy is typically used to affect responses in the bone marrow and/or lymphatic compartments.

Administration of Cyanoaziridines

In addition to intravenous (systemic) therapy, with some cancers, drugs may need to be given directly into the central nervous system since they have a low uptake into this compartment. Therefore, the cyanoaziridines may be injected through the intrathecal space between the third and fourth lumbar vertebrae in order to achieve high levels in the cerebral spinal fluid. Alternatively, the drugs may be injected through a subcutaneous sack connected to the brain ventricle (the Ommaya reservoir). In this way the cyanoaziridines can gain access to the central nervous

system to reduce tumor cell burden in that compartment. Drugs useful in the central nervous system generally have low sclerosing or vesicant potential; current examples include the antimetabolites cytarabine and methotrexate. The cyanoaziridines also have a low sclerosing and vesicant potential.

Drug Administration

For a typical therapeutic use of imexon to treat a systemic cancer not in the central nervous system the drug is dissolved in a physiologic solution such as 5% dextrose in water (D5W) or 0.9% sodium chloride for injection (normal saline). This solution is then infused intravenously either via a peripheral vein or a central vascular access device at a slow infusion rate of several mL/minute. The infusion may be continued for several days or it may be given in a single daily injection over a period of 30 minutes to 4 hours. Throughout this infusion the patient is monitored for any acute distress such as a hypersensitivity reaction to the drug or a change in blood pressure or mental status. The veins are also monitored to make sure that medication is not leaking out (extravasation). At the conclusion of the infusion the patient may remain under observation for a short time period before returning home or to work.

The range of imexon (analog) doses may vary from 10 mg to 10 grams per day for single or consecutive daily doses. Prolonged daily dosing has been shown to be tolerable and active in tumor-bearing mice and dogs receiving therapy for up to one month. Thus, a "typical" clinical dose schema for humans with cancer would comprise: 1-10 g/day by IV infusion for one up to thirty consecutive days, followed by a "rest period" of 1-2 weeks, for evaluation of response and resolution of any toxicities, which are expected to involve non-cumulative gastrointestinal effects. The rates of infusion are typically 1-500 μg (generally 100-300) per mL per m^2 at rates of 1-500 (typically 100-300) mg/hour. These treatment schedules could be continued indefinitely until there is evidence of disease progression or severe toxicity.

Table 1A shows the designation numbers of imexon analogs (AMPs) and their respective chemical formulae. When an AMP designation number is used herein, its chemical formula can be found in Table 1A.

Table 1A. AMP designation of imexon analogs and their respective chemical formulae.

compound	R
AMP-400	(imexon)
403	CH_3
404	C_6H_5
405	$COCl_3$
406	C_2H_5
407	o,p-$C_6H_3Cl_2$
408	pm,p-$C_6H_3Cl_2$
409	H (intermed.)
410	$C(CH_3)_3$
412	$CH_2C_6H_5$
413	c-C_6H_{11}
414	C_4H_9
415	p-FC_6H_4
416	p-$CF_3C_6H_4$
417	p-$O_2NC_6H_4$
418	Bis-cyanoaziridine

419	p-$C_2H_5OCOC_6H_4$
420	$C_2H_5OCOCH_2$
421	C_5H_4N (pyr.)
422	$H_2NSO_2C_6H_4$
423	l-Naphthyl
424	o-$CH_3CO_2C_6H_4$
425	m-$CH_3COC_6H_5$

Table 1B below summarizes the projected clinical uses for the imexon analog series. Based on the current preclinical data, the imexon analogs will require parenteral administration by the intramuscular, intravenous or subcutaneous route. A depot (IM or SC) formulation would be especially advantageous to prolong drug levels and reduce injection frequency. The parent compound was not active orally in mice, but select analogs will overcome this limitation due to their enhanced stability in acidic aqueous solutions, and/or by incorporation into an oral formulation which is protected from gastrointestinal degradation (i.e., enteric coatings or other timed-release oral formulations.

Table 1B. Clinical Uses of the Imexon Analog Series in Treating Human Cancer.

Disease	Rationale
Multiple Myeloma	• Activity demonstrated in human tumors *in vitro* and *in vivo* (in the SCID mouse model)
Lung Cancer, Breast Cancer	• Parent compound active in Phase I human clinical trial

Malignant Malanoma	• Human cells sensitive to analogs in *in vitro* and parent compound active in Phase I human clinical trial and *in vivo* in animal AIDS-related lymphoma models
AIDS-Related Lymphoma	• Parent compound active *in vitro* • Analogs active against lymphoma cells *in vitro*
Multidrug-Resistant (MDR) Tumors (Myeloma, Leukemia Breast and Colon Carcinoma)	• Analogs not affected by p-glycoprotein-mediated MDR
Prostate Cancer	• Parent compound active in human tumors *in vivo* in the SCID mouse model

Management of Cyanoaziridine Toxicities and Responses

Based on the *in vitro* and *in vivo* observations of enhanced and tumor efficacy for prolonged exposure, a clinical trial design to incorporate this requirement, has been formulated for imexon and imexon-related analogs. Our prior studies of imexon in mice and dogs suggest that the maximally-tolerated human dose will be approximately 500 mg/m^2/day. (Note an "average" adult is approximately 1.8 m^2 in body surface area.)

It is anticipated that the major toxicity with the cyanoaziridines will be acute nausea and vomiting and these will be pretreated with combinations of effective antiemetics. In addition, several weeks after the conclusion of the cyanoaziridine derivative infusions the patients will have blood work drawn to evaluate both toxic effects on different normal tissues including the bone marrow, kidney and liver as well as evidence for reduction in any of the known tumor markers as described earlier. In general the patients are reassessed on a monthly basis and therapy may be then reinstituted if there is no evidence of tumor growth and/or if there is evidence of actual response to the prior therapy as described earlier. These treatments may be combined with other anticancer agents given on a cyclical basis of usually every 2-4 weeks with approximately 5-6 courses typically given in order to

comprise a complete trial of chemotherapy. At this time the patient is completely reassessed for response and toxicity and patients may either continue on therapy if there is some evidence of residual disease or they may go into a period of observation if the tumor has been significantly reduced. With any increasing sign of tumor spread patients may go back onto chemotherapy with the cyanoaziridine and/or other agents or a decision may be made to switch to different types of drugs and/or modality such as radiation therapy.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

EXAMPLE 1

2-Cyanoaziridine-1-(N-methyl)carboxamide

To an ice-cold mixture of 2-cyanoaziridine and toluene was added an ice-cold solution of methyl isocyanate (1.05 equivalents) in toluene at a rate to keep the temperature below 5°C. The mixture was stirred for one hour in an ice bath and then placed in a refrigerator overnight. The resulting precipitate was collected, washed with toluene, and dried under vacuum to give a 94% yield of the title compound as a solid with m.p. 98-100 °C: MS (EI) 125(M^+); ^1H NMR ($CDCl_3$, TMS) 2.47 (d, 1, J=3 Hz), 2.57 (d, 1, J=6 Hz) 2.8 (d, 3, J=5 Hz), 3.05 (2d, 1, J=6 Hz, 3 Hz) 6.18 (s, 1, NH).

EXAMPLE 2

2-Cyanoaziridine-1-(N-ethyl)carboxamide

This compound was prepared from 2-cyanoaziridine and ethylisocyanate in 63% yield by the procedure described in Example 1. It had m.p. 58-62 °C: MS (EI) 139 (M^+); ^1H NMR ($CDCl_3$, TMS) 1.1 (t, 3, J=6 Hz), 2.4 (d, 1, J=3

Hz), 2.50 (d, 1, J=6 Hz), 2.97 (2d, 1, J=6 Hz, 3 Hz), 3.3 (q, 2, J=6 Hz), 6.1 (s, 1, NH).

EXAMPLE 3

2-Cyanoaziridine-1-(N-butyl)carboxamide

This compound was prepared from 2-cyanoaziridine and *n*-butylisocyanate in 92% yield by the procedure described in Example 1. It had m.p. 32-34 °C: ^{1}H NMR ($CDCl_3$, TMS) 1.0 (t, 3), 1.2-1.5 (m, 2), 1.6-2.1 (m, 2), 2.5 (d, 1, J=3 Hz), 2.55 (d, 1, J=6 Hz), 3.19 (2d, 1, J=6 Hz, 3Hz), 3.6 (m, 2), 5.8 (s, 1, NH).

EXAMPLE 4

2-Cyanoaziridine-1-(N-t-butyl)carboxamide

This compound was prepared from 2-cyanoaziridine and *t*-butylisocyanate in 81% yield by the procedure described in Example 1. It had m.p. 46-48 °C: ^{1}H NMR (DMSO-d_6, TMS) 1.4 (s, 9), 2.84 (d, 1, J=3 Hz), 2.86 (d, 1, J=6 Hz), 3.0 (2d, 1, J=6Hz, 3Hz) 5.8 (br. s, NH).

EXAMPLE 5

2-Cyanoaziridine-1-(N-cyclohexyl)carboxamide

This compound was prepared from 2-cyanoaziridine and cyclohexylisocyanate in 64% yield by the procedure described in Example 1. It had m.p. 98-102 °C: ^{1}H NMR ($CDCl_3$, TMS) 1.2-1.5 (m, 5), 1.6-2.1 (m, 5), 2.45 (d, 1, J=3 Hz), 2.53 (d, 1, J=6 Hz), 3.015 (2d, 1, J=6 Hz, 3 Hz), 3.6 (m,1), 5.8 (s, 1, NH).

EXAMPLE 6

2-Cyanoaziridine-1-(N-benzyl)carboxamide

Method A. This compound was prepared from 2-cyanoaziridine and benzylisocyanate in 25% yield by the procedure described in Example 1. It had m.p. 42-44 °C: ^{1}H NMR ($CDCl_3$, TMS) 2.36 (d, 1, J=3 Hz), 2.46 (d, 1, J=6 Hz), 2.93 (2d, 1, J=6 Hz, 3 Hz), 4.4 (d, 2), 6.8 (s, 1, NH), 7.4 (m,5).

Method B. A solution of 175 mg of 2-cyanoaziridine and 0.36 mL of triethylamine in 5 mL of THF was cooled and stirred in an ice bath. A solution of 550 mg of 4-Nitrophenyl chloroformate in 2.5 mL of THF was added at a rate that kept the temperature below 10 °C. When the addition was complete, the solution was stirred 2 hours at room temperature and then filtered to remove triethylamine hydrochloride. The filtrate was concentrated under reduced pressure and the residual oil was stirred with 5 mL of toluene for 30 minutes. The pale yellow precipitate that formed was washed with toluene (2 x 5 mL) and dried under vacuum to afford a 33% yield of 4-nitrophenyl 2-cyanoaziridine-1-carboxylate, m.p. 100-104 °C.

A mixture of 4-nitrophenyl 2-cyanoaziridine-1-carboxylate and benzylamine (1:1.1 molar ratio) in THF was stirred vigorously at room temperature. The progress of the reaction was monitored by thin-layer chromatography on silica gel with chloroform-methanol (1:9) as solvent. When the starting materials were consumed (about 2 hours), the solution was concentrated under reduced pressure and the title compound was freed from 4-nitrophenol by washing with THF. This procedure gave the title compound with m.p 42-44 °C and a ^1H NMR spectrum identical with that of the sample described in Method A.

EXAMPLE 7

2-Cyanoaziridine-1-[N-(ethoxycarbonyl)methyl]carboxamide

This compound was prepared from 2-cyanoaziridine and ethyl isocyanatoacetate in 50% yield by the procedure described in Example 1, except that it was a colorless oil and did not crystallize on refrigeration. Instead, it was dissolved in cold chloroform and diluted with cold hexane. The mixture was stirred briefly and then the solvent was decanted. The residual title compound had ^1H NMR ($CDCl_3$, TMS) 1.22 (t, 3, J=7 Hz), 2.49 (d, 1, J=3 Hz), 2.56 (d, 1, J=6 Hz), 3.07 (2d, J=6 Hz, 3 Hz), 3.91 (d, 2), 4.15 (q, 2, J=7 Hz), 6.74 (t, 1, NH).

EXAMPLE 8

2-Cyanoaziridine-1-(N-phenyl)carboxamide

This compound was prepared from 2-cyanoaziridine and phenylisocyanate in 71% yield by the procedure described in Example 1. It had m.p. 88-90 °C: MS (EI) 187(M^+); ^{1}H NMR (DMSO-d_6, TMS) 2.65 (d, 1, J=3 Hz), 2.69 (d, 1, J=6 Hz), 3.57 (2d, 1, J=6 Hz, 3 Hz), 7.05 (t, 1), 7.45 (d, 2), 7.60 (d, 2), 10.2 (s, 1, NH).

EXAMPLE 9

2-Cyanoaziridine-1-[N-(4-fluorophenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and (4-flourophenyl)isocyanate in 54% yield by the procedure described in Example 1. It had m.p. 99-100 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.55 (d, 1, J=3 Hz), 2.68 (d, 1, J=6 Hz), 3.20 (2d, 1, J=6 Hz, 3 Hz), 7.0 (d, 2, J=9 Hz), 7.5 (d, 2, J=9 Hz), 10.2 (s, 1, NH).

EXAMPLE 10

2-Cyanoaziridine-1-[N-(4-trifluorophenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and 4-(trifluorophenyl)isocyanate in 91% yield by the procedure described in Example 1. It had m.p. 166-168 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.62 (d, 1, J=3 Hz), 2.74 (d, 1, J=6 Hz), 3.32 (2d, 1, J=6 Hz, 3 Hz), 7.54 (d, 2, J=9 Hz), 7.74 (d, 2, J=9 Hz), 10.2 (s, 1, NH).

EXAMPLE 11

2-Cyanoaziridine-1-[N-(4-nitrophenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and 4-(nitrophenyl)isocyanate in 89% yield by the procedure described in Example 1. It decomposed above 230 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.77 (d, 1, J=3 Hz), 2.81 (d, 1, J=6 Hz), 3.69 (2d, 1, J=6 Hz, 3 Hz), 7.8 (d, 2, J=9 Hz), 8.2(d, 2, J=9 Hz), 10.8 (s, 1, NH).

EXAMPLE 12

2-Cyanoaziridine-1-[N-(2,4-dichlorophenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and (2,4-dichlorophenyl)isocyanate in 50% yield by the procedure described in Example 1. It had m.p.110-114 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.70 (d, 1, J=3 Hz), 2.71 (d, 1, J=6 Hz), 3.57 (2d, 1, J=6 Hz, 3 Hz), 7.4 (d, 1, J=6 Hz), 7.6 (d, 1, J=6 Hz), 7.7 (s, 1), 10.0 (s, 1, NH).

EXAMPLE 13

2-Cyanoaziridine-1-[N-(3,4-dichlorophenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and (3,4-dichlorophenyl)isocyanate in 76% yield by the procedure described in Example 1. It had m.p. 132-134 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.71 (d, 1, J=3 Hz), 2.73 (d, 1, J=6 Hz), 3.62 (2d, 1, J= 6Hz, 3 Hz), 7.5 (2d, 1, J=9 Hz, 3 Hz), 7.6 (d, 1, J=9Hz), 7.9 (d, 1, J=3 Hz), 10.6 (s, 1, NH).

EXAMPLE 14

2-Cyanoaziridine-1-[N-(4-ethoxycarbonylphenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and ethyl 4-isocyanatobenzoate in 90% yield by the procedure described in Example 1. It had m.p.162-165°C: ^{1}H NMR (DMSO-d_6, TMS) 1.3 (t, 3, J=6 Hz), 2.72 (d, 1, J=3 Hz), 2.76 (d, 1, J=6 Hz), 3.64 (2d, 1, J=6 Hz, 3 Hz), 4.3 (q, 2, J=6 Hz), 7.69 (d, 2, J=9 Hz), 7.73 (d, 2, J=9 Hz), 10.63 (s, 1, NH).

EXAMPLE 15

2-Cyanoaziridine-1-[N-(3-acetylphenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and (3-acetylphenyl)isocyanate in 74% yield by the procedure described in Example 1. It had m.p. 110-112 °C: ^{1}H NMR (DMSO-d_6, TMS) 2.6(s, 3), 2.71 (d, 1, J=3 Hz), 2.74

(d, 1, J=6 Hz), 3.63 (2d, 1, J=6 Hz, 3 Hz), 7.5 (t, 1, J=9 Hz), 7.7 (d, 1, J=9 Hz), 7.85 (d, 1, J=9 Hz), 8.1 (s, 1), 10.5 (s, 1, NH).

EXAMPLE 16

2-Cyanoaziridine-1-[N-(2-acetoxyphenyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and (2-acetoxyphenyl)isocyanate in 10% yield by the procedure described in Example 1. It had m.p. 100-102 °C: ^{1}H NMR ($CDCl_3$, TMS) 2.38 (s, 3), 2.55 (br. s, 1), 2.64 (br. s, 1,), 3.20 (br. s, 1), 7.15 (br. s, 2), 7.2-7.6 (br. s, 1), 7.68 (br. s, 1), 7.96 (s, 1, NH). In this spectrum, the expected doublets were not resolved, but appeared as broad singlets.

2-Acetylbenzoic acid azide was prepared by reacting 2-acetoxybenzoyl chloride with sodium azide in acetone and water at 0-5 °C for 24 hours. It had an IR peak at 2245 cm^{-1} (azide). The crude azide was then heated in benzene at 70-75 °C under nitrogen for 2 hours to give 2-acetoxyphenylisocyanate.

EXAMPLE 17

2-Cyanoaziridine-1-[N-(4-sulfamylphenyl)]carboxamide

2-Cyanoaziridine-1-[N-(4-chlorosulfonyl)]carboxamide was prepared from 2-cyanoaziridine and (4-chlorosulfonylpheny)isocyanate by the procedure described in Example 1. It had m.p. 142-144 °C. Without further purification, it was converted by treatment with liquid ammonia into the title compound, in overall yield of 39%: m.p. 170-174 °C; ^{1}H NMR (DMSO-d_6, TMS) 2.72 (d, 1, J=3 Hz), 2.74 (d, 1, J=6 Hz), 3.7 (2d, 1, J=6 Hz, 3 Hz), 7.26 (s, 2, SO_2NH_2), 7.69 (d, 2), 7.73 (d, 2), 10.6 (s, 1, $CONH_2$).

EXAMPLE 18

2-Cyanoaziridine-1-[N-(1-naphthyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and 1-naphthylisocyanate in 56% yield by the procedure described in Example 1. It had

m.p. 98-100 °C: ^{1}H NMR ($CDCl_3$, TMS) 2.6 (br. s, 1), 2.7 (br. s, 1), 3.2 (br. s, 1), 7.4 (br. s, 1), 7.5 (m, 2), 7.7 (br. s, 2), 7.8 (br. s, 2), 8.1 (s, 1, NH).

In this spectrum, the expected doublets were not resolved and appeared as broad singlets.

EXAMPLE 19

2-Cyanoaziridine-1-[N-(3-pyridyl)]carboxamide

This compound was prepared from 2-cyanoaziridine and 3-pyridylisocyanate by the procedure described in Example 1, except that the solvent was benzene. It had m.p. 205 °C (dec.): ^{1}H NMR (DMSO-d_6, TMS) 2.72 (d, 1, J=3 Hz), 2.76 (d, 1, J=6 Hz), 3.65 (2d, 1, J=6 Hz, 3Hz), 7.36 (d, 1), 7.97 (d, 1), 8.27 (d, 1), 8.71 (s, 1, NH), 10.5 (br. 2, 1, NH).

3-Pyridylisocyanate was prepared from nicotinic acid hydrazide by way of nicotinic acid azide following the literature procedure. (Hyden, *et al.*, *Chem Ind* (London) 3:1406-1407, 1967.) It had an IR peak at 2250 cm^{-1} (isocyanate). The overall yield of the title compound was 10%.

EXAMPLE 20

In vitro and *in vivo* model systems for testing the compounds of this invention.

The compounds of the present invention were tested for antitumor activity in various model systems. These systems included the following:

1) *in vitro* tumor cell viability assays using MTT and SRB dye reaction endpoints.

2) *in vivo* survival studies in mice with severe combined immunodeficiency disease (SCID) bearing solid flank tumors.

Sulforhodamine B (SRB) Assay for Adherent Tumor Cells

Background: Sulforhodamine B (SRB) is a protein binding aminoxanthene dye with two sulfonic acid groups. (Lillie, R.D., *H.J. Conn's Biological Stains*, 9th Ed., Baltimore; Williams & Wilkins, 1977.) It quantitates whole

cell protein content by the intensity of dye staining and relates this to the number of viable cells. This assay assumes that dead cells either lyse, are removed during the procedure, or otherwise do not contribute to the colorimetric endpoint. The SRB assay is used for cells which normally adhere to surfaces (in this case plastic culture flasks or petri dishes) as part of their requirement for growth and division *in vitro*. The SRB assay is currently used by the National Cancer Institute in the anticancer drug screening program. (Skehan, *et al., J Natl Cancer Inst* 82:1107-1112, 1990; Rubinstein, *et al., J Natl Cancer Inst* 82:1113-1118, 1990.) Methodologic comparisons have shown that SRB results are linear for the number of tumor cells over a 100-fold range, and for protein content determined by a modified Lowry method or Standard Bradford (Bradford, M., *Anal Biochem* 72:248-254, 1976) methods. The visible pink colorimetric endpoint is also indefinitely stable and is more sensitive for quantitating cell numbers than the Lowry or Bradford colorimetric methods and is comparable in sensitivity to fluorescent detection methods. (Lillie, R.D., *H.J. Conn's Biological Stains*, 9th Ed., Baltimore; Williams & Wilkins, 1977.)

Methodology: Cell cultures of 10^3-10^7 cells growing in 96-well plastic microtiter plates are fixed with 50% trichloroacetic acid (TCA) for 30 minutes at 4 °C. The cells are then stained with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye is removed by 4 washes with 1% acetic acid, and protein-bound dye is extracted with 10 mM unbuffered Tris® base [tris(hydroxymethyl)aminomethane]. The optical density (OD) of this extracted solution is measured at 564 nM in a microtiter plate ultraviolet absorbance detector. Cell viability is expressed as percent of control by dividing the optical densities and multiplying the result by 100.

Microculture Tetrazolium (MTT) Assay for Tumor Cells

Tumors are disaggregated into single cell suspensions using mechanical, hypoosmotic, and/or enzymatic (trypsin) methods. The cells are plated at a concentration of 3-5 x 10^4 per 1 mL well into plastic 96-well plates. Growth medium containing 5-10% (vol/vol) heat-inactivated fetal bovine serum and penicillin/streptomycin (both 100 mg/mL) are added prior to incubation at 37 °C for 6

days. Afterwards the medium containing the drug is removed, the cells are centrifuged in fresh medium or phosphate-buffered saline (pH 7.4). A tetrazolium dye, (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), is then added. (Mossman, T., *J Immunol* 65:55-63, 1983.) This dye forms a colored formazan product upon activation by mitochondrial reductases in viable cells. Typically, the formazan product is solubilized in acid-propanol or DMSO. The intensity of the color is proportional to viable cell numbers and this is quantitated by spectrophotometric absorbance at 570 nM on a micro ELISA plate reader. The results are calibrated in % control absorbance from untreated tumor cells. (Alley, *et al.*, *Cancer Res* 48:589-601, 1988.)

Testing for Antitumor Efficacy in Mice with Severe Combined Immunodeficiency Disease (SCID)

Background: The SCID mouse represents an autosomal recessive mutation in an inbred strain of C_1B-17 mice which renders the animals profoundly immunodeficient and able to accept tissue xenografts from rats, humans or other strains of mice. (Ware, *et al.*, *J Immunol Methods* 85:353-361, 1985.) The immunologic defect results from an inability to carry out normal immunoglobulin and T-cell receptor recombination reactions during the development of T- and B-lymphocytes. This renders mice with very low levels of T- and B-cells, and low levels of antibody production which allows foreign (allogeneic or xenogeneic) tissue grafts to grow *in vivo*. A variety of human tumor tissue types have been shown to grow into palpable tumors following subcutaneous injection into SCID mice. (Phillips, *et al.*, *Curr Top Microbiol Immunol* 152:259-263, 1989.) Compared to athymic nude mice, SCID mice accept more tumor tissue types and breed more efficiently. (Phillips, *et al.*, *Curr Top Microbiol Immunol* 152:259-263, 1989.) The SCID mice are also more sensitive to ionizing radiation (Budach, *et al.*, *Cancer Res* 52(22):6292-6296, 1992) due to a defect in DNA double strand break repair. (Chang, *et al.*, *Cancer Res* 53:1244-1248, 1993.) Because some SCID mice can spontaneously revert to a more normal phenotype, serum immunoglobulin G (IgG) levels are

routinely measured and if elevated (leaky phenotype syndrome), the mouse is excluded from further study.

Methodology: The SCID mouse colony is maintained in a barrier facility with specially filtered air, autoclaved (sterilized) food and water and protective handling precautions (gloves, mask, gown) to prevent inadvertent infection. For tumor studies, human tumor cells are grown *in vitro*, harvested, counted and injected subcutaneously into the front flank area. Tumor cell innocula range from 10^6-$2x10^7$ cells/mouse. Palpable tumors begin to develop after 1-3 weeks and sizes are determined by caliper measure of the widest perpendicular diameters. These dimensions are converted to mass using the formula: $(L \times W^2) \div 2$

wherein W = the shorter of the two dimensions and L = the longer diameter value.

Tumor growth rates are plotted as mass (g) per days after inoculation.

Drug treatments are given by intravenous (IV) or intraperitoneal (IP) injection beginning 24 hours after tumor implantation (day 1). For imexon analog 404 studies, 20×10^6 8226 myeloma cells exhibiting 40-fold resistance to doxorubicin (8226) (Dalton, *et al.*, *Cancer Res* 46:5125-5130, 1986) were injected in SCID mice (5/group) on day 0. Drug therapy began 1 day later with AMP-404 dissolved in a water-miscible co-solvent system containing (per mL): benzyl alcohol 30 mg; polysorbate 80 mw, 80 mg; polyethylene glycol 300 mw, 650 mg; and q.s. to 1.0 mL in absolute ethanol. The daily dose was either 50 mg/kg or 100 mg/kg. Therapy was continued daily for 4 weeks and tumors were measured three times per week up to the 7 week ending period (two weeks after stopping drug therapy).

Results with AMP-404 in SCID Mice Bearing Human 8226 Myeloma Tumors

Daily treatment with AMP-404 100 mg/kg resulted in a reduced rate of tumor growth. This was statistically significant at 3 weeks. At the end of the 6 week experiment, mean control tumor size was approximately 1,000 mm^3 in the control group and in the group treated with 100 mg/kg it was approximately 300 mm^3 (Table 2). This group also experienced a mean body weight loss of 15-20% compared to

the control group. There was no apparent weight loss or antitumor effect in the group of SCID mice treated with 50 mg/kg of AMP-404. The tumor take rate (% of inoculated mice developing palpable tumors) was 100% in all 3 groups. The onset of developing palpable tumors was 3 weeks after inoculation. The results are provided in Figure 1. These results show that AMP-404 is active against multidrug-resistant human myeloma cells *in vivo*. Antitumor effects at the 100 mg/kg dose were associated with a mean 50% reduction in tumor volume. There was no drug-induced lethality, but this regimen did reduce total body weight by 15-20%. The lower AMP-404 dose of 50 mg/kg was not active.

Table 2: Effect of AMP-404 on Human 8226 Myeloma Tumor Growth in SCID Mice

Group (Dose)	No. Mice	Mean (SD) Tumor Volume in mm^3				
		Week 3	Week 4	Week 5	Week 6	Week 7
Control (Solvent)	5	109.6 (37.9)	311.9 (204.3)	530.4 (339.6)	1,042.1 (794.1)	1,891 (1,692.3)
AMP-404 (50 mg/kg)	5	104.2 (78.6)	247.9 (225.9)	574.2 (296.2)	1,020 (396.2)	1,858.4 (941.4)
AMP-404 (100 mg/kg) (P-value)*	5	32.9 (17.9)*	104.9 (81.8)	312.5 (195.5)	357.3 (314.5)	824.5 (793.7)
(P-Value)*		(.005)	(.068)	(.229)	(.100)	(0.19)

* Unpaired T-test compared to control group.

Results with Aziridine-1-carboxamides in Tumor Cell Lines *In Vitro*

Table 3 gives the potencies of the new aziridine-1-carboxamides in a panel of human and murine tumor cells in culture. The tumor cell lines in this table are selected to represent a range of histological and growth phenotypes. Their characteristics are described below.

According to Table 3, the compounds of this invention are active at low concentrations against human breast carcinoma cells in culture, including cell lines resistant to the important clinical agents doxorubicin and mitoxantrone. They show little or no cross-resistance in these cell lines. Furthermore, they are active at comparable low concentrations against colon cancer, lung cancer, and ovarian cancer cell lines.

Table 3: Antitumor Activities of Aziridine-1-carboxamides in Human Solid Tumor Cell Lines

		IC_{50}(μg/mL)						
		MCF-7 Breast			WiDr colon	A-549 lung	A-375 melanoma	OVCAR3 ovarian
AMP No.	Example No.	sens.	D40	mitox.				
403	1	3.3	2.1	2.3			2.1	1.7
404	8	0.5	0.5	0.8	0.9	1.6	2.1	1.3
406	2	4.0	2.8	1.4	5.6	2.5	5.0	2.0
407	12						2.0	0.6
410	4	>10	31.8	20.1	30	33	69.9	31.1
412	6	1.5	1.2	0.7	1.1	1.5	2.2	1.0
413	5	2.5	1.7	1.9	0.4	1.9	6.7	5.9
414	3						2.5	2.0
415	9	0.4	0.5	0.6	0.4	1.3	0.7	1.2
416	10	0.4	0.4	0.3	0.3	1.1	3.0	1.5
417	11						0.9	1.5
419	14					1.5	2.0	1.5
420	7						10	10
421	19						2.3	1.9
422	17						50	50
423	18						1.0	1.4
424	15						>10	>10
425	16						1.8	2.23

Sens. = Sensitivity to doxorubicin
D 40 = 40-fold doxorubicin resistant
Mitox = Mitoxantrone resistant pb

Description of Cell Lines Used to Characterize Antitumor Activity of Imexon Analogs *In Vitro*

1.0 Human Breast Tumor Cell Lines

1.1 MCF-7 Breast Cancer: The parental (sensitive) MCF-7 breast cancer is an estrogen-dependent adenocarcinoma originally derived from a pleural effusion in a female with advanced metastatic breast cancer. (Soule, *et al., J Natl Cancer Inst* 51:1409-1416, 1973.)

1.2 MCF-7/ADR: This multidrug-resistant MCF-7 cell line was developed by growth in medium containing serially increasing concentrations of the antitumor agent doxorubicin (Adriamycin®). (Cohen, *et al., Cancer Res* 46:4087-4090, 1986.) The resistance pattern is stable when the cells are grown in medium of doxorubicin and the cells are cross-resistant to numerous other natural product-based antitumor agents including vincristine, vinblastine, etoposide, and dactinomycin. The MCF-7 ADR line is 192-fold less sensitive to doxorubicin *in vitro* and is known to express the cell membrane efflux (resistance) pump, P-glycoprotein.

1.3 MCF-7/D40 and MCF-7/MITOX cell lines were developed by chronic exposure *in vitro* to increasing concentrations of the DNA-intercalating antitumor agents doxorubicin and mitoxantrone, respectively. (Taylor, *et al., Br J Cancer* 63:923-929, 1991.) These resistant cell lines have similar growth characteristics to the parental MCF-7 cells, but exhibit a multidrug resistance phenotype. The MCF-7/D40 cells express the cell membrane efflux pump, P-glycoprotein and are over 40-fold resistant to doxorubicin, mitoxantrone and the vinca alkaloids, vinblastine and vincristine. In addition, MCF-7/DOX40 cells can be sensitized (resistance reversed) using the antiarrhythmic agent verapamil. The MCF-7/MITOX cells are similarly multidrug resistant (but do not express either P-glycoprotein or the MRP resistance protein) (Futscher, *et al., Biochem Pharmacol* 47(9):1601-1606, 1994), and resistance is not modulated (reversed) by verapamil. Furthermore, the MCF-7/MITOX cells are only partially resistant to doxorubicin, but are highly resistant to other natural products.

2.0 Human WiDr Colon Cancer Cell Lines

2.1 WiDr: The adenocarcinoma cell line WiDr was originally isolated by Noguchi, *et al.* from a primary colon tumor specimen from a patient with advanced colon carcinoma. (Noguchi, *et al, In Vitro* 35(6):401-407, 1979.) The WiDr MITOX cell line was developed for mitoxantrone resistance by growth in serially elevated concentration of mitoxantrone. (Wallace, *et al.*, *Proc Am Assoc Cancer Res* 23:767, 1982.) It is P-glycoprotein negative and exhibits 21-fold resistance to mitoxantrone, 8-fold resistance to doxorubicin and only 2-fold resistance to vincristine. (Dalton, *et al.*, *Cancer Res* 48:1882-1888, 1988.)

3.0 A-549 Human Lung Cancer is an adenocarcinoma type of non-small cell lung cancer (NSCLC). It was established by Girard, *et al.* from a fresh tumor specimen obtained from a 58 year-old male with advanced lung cancer. (Girard, *et al.*, *J Natl Cancer Inst* 51(5):1417-1423, 1973.) It has 5.6% plating efficiency (colony formation) on agar-coated plates, 66 modal chromosome number and grows rapidly when injected subcutaneously, forming acinar pattern tumors in immunodeficient mice. (Girard, *et al.*, *J Natl Cancer Inst* 51(5):1417-1423, 1973.) Like NSCLC *in vivo*, A-540 lung cancer cells are relatively resistant to most chemotherapy drugs *in vitro* but like MCF-7 and WiDr, is used to screen for new anticancer agents in the NCI *in vitro* screening planel. (Alley, *et al.*, *Cancer Res* 48:589-601, 1988.)

4.0 The Human A-375 Malignant Melanoma Cell Line was also developed by Girard, *et al.* (Girard, *et al.*, *J Natl Cancer Inst* 51(5):1417-1423, 1973) from a 54 year-old human female with advanced melanoma. The modal chromosome number is 67 and it has a low plating (colony forming) efficiency of 0.7% when grown on agar. These cells also form rapidly growing tumors when injected subcutaneously into immunodeficient athymic (nu/nu) mice. (Girard, *et al.*, *J Natl Cancer Inst* 51(5):1417-1423, 1973.) These cells are also used in the NCI drug screening panel. (Skehan, *et al.*, *J Natl Cancer Inst* 82:1107-1112, 1990.)

5.0 Human OVCAR-3 Ovarian Carcinoma was originally isolated by Hamilton, *et al.* Experimental model systems of ovarian cancer; applications to the design and evaluation of new treatment approaches. *Seminars in Onocology*, 11:285-298, 1984, from a patient with progressive adenocarcinoma of the ovary. This patient had relapsed after receiving a combination chemotherapy regimen containing cyclophosphamide, doxorubicin and cisplatin. The cells grow as a cobblestone-like monalayer on plastic, have several chromosomal abnormalities, and possess intracellular hormonal receptors for androgens and estrogens. The OVCAR-3 cell line is resistant *in vitro* to clinically relevant concentrations of doxorubicin, melphalan and cisplatin. It is also one of the standard human tumor cell lines used for screening new anticancer agents at the NCI. (Alley, *et al.*, *Cancer Res* 48:589-601, 1988.)

6.0 Development and Characterization of an Imexon-Resistant Human 8226 Myeloma Cell Line

The following unpublished studies were performed *in vitro* to develop and characterize a human myeloma cell line with resistance to imexon-induced cytotoxicity. The original (parental) 8226 cell line was developed by Matsuoka, *et al.* (Matsuoka, *et al.*, *Proc Soc Exp Biol Med* 125:1246-1250, 1967). It is a lymphoblastoid cell line which stains for lambda light immunoglobulin production. It grows *in vitro* in suspension culture (non-adherent) and forms tumors when injected into SCID mice. (Skehan, *et al.*, *J Natl Cancer Inst* 82:1107-1112, 1990.)

EXAMPLE 21

Selection of Imexon-Resistant Cells and Cell Growth Characteristics

One exciting feature of our novel compounds is that they work on imexon-resistant cells. An imexon-resistant 8226 myeloma cell line was selected by continuously exposing cells to gradually increasing concentrations of imexon up to 3.25 mg/mL. This cell line has slightly longer cell doubling time than 8226 sensitive cells (24 vs 20 hours). The survival curves for 8226 imexon-resistant cells (8226/I-R) and 8226 sensitive cells (8226/S) in the presence of imexon are showed in Figure 2 which provides dose response curves for imexon on 8226

myeloma cells. The dose response curve for imexon continuous exposure was determined using MTT assay. Each data point represents the mean of three experiments performed in 8 replicates.

We compared IC_{50} concentrations and there is a 4-fold resistance observed for the 8226/I-R cell line when the cells were continuously exposed to imexon for 5 days at 37 °C. The degree of resistance was relatively unstable in the absence of the drug, with the resistant cells losing substantial resistance when maintained in imexon-free medium for 20 weeks (Figure 2).

Cytogenetic Studies: The range of chromosome counts was 48-79 in 8226/S and 51-62 for 8226/I-R with modal populations of 57-59 (59%) and 61 (40%). Both cell lines had 12 identifiable structural abnormalities, including dic(1;14), t(1;14)(p13;q32), add(3)(q29), del(5)(q32), der(5)t(5;6), der(7)t(3; 7), add(9)(p24), del(11)(q24), del(12)(p11.2), add(16)(q24), der(17) t(11; 17) and 21 qhsr. The imexon-resistant cell line had two additional structure rearrangements that are del(1)(p22) and add(6)(q13). The guanine-banded karyotype of 8226/S and 8226/I-R cells was shown in Figure 3. Since there was a rearrangement at der(7)t(3, 7) which was a characteristic of 8226/V cells (a cell line developed for resistance to verapamil), the 8226/S cells were actually 8226/V cells.

Cross-Resistance Study: The cross-resistance profile of imexon-resistant cells measured by MTT assay is shown in Table 4. The 8226 imexon-resistant cells showed partial cross-resistance to the platinum-type DNA intrastrand cross linkers, cisplatin and carboplatin. There is also partial resistance to the bioreductive aziridine-based alkylator mitomycin C. However, this imexon-resistant cell line remains sensitive to natural products, including doxorubicin, vincristine, and bleomycin and X-radiation.

Table 4. Cross-Resistance Patterns of Imexon-Resistant 8226 Human Myeloma Cells to Various Antitumor Agents.

Agent	IC_{50}(M) 8226/S	8226/I-R	Fold Resistance[a]
Imexon	2.7×10^{-5}	1.2×10^{-4}	4.44
Nitrogen Mustard	6.6×10^{-6}	6.0×10^{-6}	0.91
Melphalan	6.2×10^{-8}	5.3×10^{-6}	0.81
Cisplatin	1.1×10^{-6}	2.0×10^{-6}	1.85
Carboplatin	9.7×10^{-6}	1.6×10^{-5}	1.65
Mitomycin C	6.6×10^{-8}	9.9×10^{-8}	1.50
Vincristine	2.8×10^{-9}	2.4×10^{-9}	0.88
Doxorubicin	4.3×10^{-10}	3.2×10^{-10}	0.79
Bleomycin[a]	7.0 μg/ml	7.8 μg/ml	1.11
X-Ray Radiation[b]	4.75	4.6	0.97

[a] IC_{50} resistant cells/IC_{50} sensitive cells.

[b] cGy.

IC_{50} was measured by MTT assay. IC_{50} refer to the concentration by drug that results in 50% growth inhibition. A minimum of 2 assays were performed for each drug following 250 mg/mL imexon is equivalent to 6.5 cGy radiation in sensitive cells.

We then composed the IC_{50} of the novel cyanoaziridines against the 8226/S- and 8226/I-R lines. Of the 20 compounds tested, 11 were as active against the imexon-resistant as imexon-sensitive lines showing a degree of resistance of 1.3 or less (IC_{50} I-R/IC_{50}S), (Table 5).

Table 5. Comparison of Imexon Analog Activity in Parental (Sensitive) and Imexon-Resistant Human 8226 Myeloma cells.

AMP No.	Example No.	IC_{50} (μg/mL) Sensitive	Resistant	Fold Resistance of IC_{50} R/IC_{50}'s
Imexon		1.0	8.5	4.5
403	1	3.3	3.3	1.0
404	8	1.0	1.5	1.5
406	2	3.1	3.6	1.2
407	12	1.2	2.4	2.0
408	13	0.27	0.68	2.5
409 imexon intermediate		**32**	**48**	**1.5**
410	4	20	36	1.8
412	6	1.8	2.2	1.2
413	5	7.0	19	1.2
414	3	2.8	2.8	1.0
415	9	1.0	1.2	1.2
416	10	2.1	2.0	0.95
417	11	2.1	2.0	0.95
419	14	ND*	ND*	-
420	7	5.0	6.0	1.2
421	19	2.2	2.8	1.3
422	17	>100	<100	-
423	18	1.0	2.1	2.1
424	15	>100	>100	-
425	16	1.98	2.2	1.1

*ND = No data.

EXAMPLE 22

Comparison of Imexon and Analog Series in the Imexon-Resistant 8226 Myeloma Cells *in vitro*

Using the colorimetric microculture tetrazolium (MTT) assay, imexon and 25-related analogs were studied for tumor growth inhibition *in vitro*. Imexon-induced DNA single strand breaks were assessed by alkaline elution. Cells were exposed to imexon for 1 hour prior to start of experiment. The fraction of ^{14}C-labeled DNA retained on the filter is plotted against the time of elution. The cells

included parental 8226 myeloma cells and the 8226 imexon-resistant cells exposed to drug (imexon or analog) continuously for 10 days at 37 °C. At the end of the exposure period, the cells were analyzed for viability using the formazan-forming tetrazolium dye (MTT) assay. For a given dose of imexon, there are fewer strand breaks in imexon-resistant cells than in sensitive cells (statistical comparison the time at which 20% of ^{14}C remaining on the filter, $p < 0.05$, $n = 2$).

The results show that imexon induces concentration-dependent single-stranded breaks in tumor cell DNA at drug concentrations which are within the range which inhibits myeloma tumor cell growth in vitro. These strand breaks are noted by an enhanced rate of ^{14}C-DNA elution through the filters after a one-hour exposure to imexon (10-500 μg/mL) at 30°C. At high imexon concentrations of ≥100 μg/mL, over 75% of the radiolabeled DNA is eluted through the filters over the test period. By comparison, control cell DNA is highly retained (>95%) on the filter over the same 24 -hour elution period. These results suggest that imexon directly or indirectly damages tumor cell DNA at drug levels which are active *in vitro*.

In addition, the imexon analogs have also been tested in a multidrug-resistant (MDR) mouse L-1210 leukemia cell line.

Murine L-1210 Leukemia Cells were first described by Law, *et al.* from a female DBA/2 mouse exposed to topical methylcholanthrene. (Law, *et al.*, *J Natl Cancer Inst* 10:179-192, 1949.) The cells represent a lymphocytic lineage and are highly tumorigenic in syngeneic DBA/2 mice, producing lethality typically 10-14 days after inoculation. This cell line has been used extensively to characterize the antitumor activity of chemotherapeutic agents. (Goldin, *et al.*, *Cancer Res* (Cancer Chemotherapy Screening Data IX) 21:27-92, 1961.) The L-1210 cells have a model 40-41 chromosomes and a very high plating efficiency in suspension cultures *in vitro.*

L-1210 MDR Cells represent a multidrug-resistant cell line developed for resistance to the alkylating agent mitomycin C. (Dorr, *et al.*, *Biochem Pharmacol* 36(19):3115-3120, 1987.) This cell line has the same general growth characteristics of the parental line, but is cross-resistant to numerous natural-product based anticancer agents. This includes anthracyclines

such as doxorubicin (Adriamycin®) and vinca alkaloids such as vincristine or vinblastine. The L-1210 MDR cells express elevated levels of membranal P-glycoprotein and resistance can be reversed using modulators such as verapamil.

The findings are similar to those in MDR myeloma in that most of the analogs exhibit no cross-resistance in a cell line which has upregulated p-glycoprotein. The results are provided in Table 6.

Table 6. Antitumor Activity of Aziridine-1 Carboxamides in Sensitive and Multidrug-Resistant (MDR) Mouse L-1210 Leukemia Cells *in vitro*.

		IC_{50}(μg/mL)		
AMP No.	Example No.	Sensitive(s)	MDR	Fold Resistance of IC_{50}'s (MDR/S)
403	1	1.1	2.7	2.5
404	8	0.2	2.1	10.5
406	2	2.0	2.0	1.0
407	12	52.3	8.4	0.16
408	13	0.7	1.9	2.7
409	32	>10.0	>10.0	
410	4	22.0	30.7	1.4
412	6	0.8	2.1	2.6
413	5	1.4	3.1	2.2
414	3	2.0	2.0	1.0
415	9	1.5	1.2	0.8
416	10	2.0	1.8	0.9
417	11	0.6	0.6	1.0
419	14	2.5	3.0	1.2
420	7	2.5	2.5	1.0
421	19	2.3	2.1	0.9
422	17	>10	>10	1.0
423	18	1.7	0.64	0.4
424	15	>10	>10	1.0
425	16	2.2	1.8	0.8

*Drug continuously present for 8 days; IC_{50} measured by MTT dye reduction assay.

Significance: These results show that (1) analogs with enhanced potency (> 40% decrease in IC_{50}) against myeloma cell growth can be identified (Nos. 407, 415, 423 and especially, 408); (2) there is a lack of significant (> 50% IC_{50} change) cross-resistance with imexon for many analogs (Nos. 403, 404, 412, 414, 415, 416, 417, 419, 420, 421 and 425); and conversely (3) some analogs have no antitumor effects even at high concentrations in the parental and imexon-resistant myeloma cells (Analog Nos. 422 and 424); and finally, (4) analogs with roughly equal potency to imexon and no significant cross resistance have been identified (Analog Nos. 412, 415, 416, 417 and 425). These results suggest that improved antitumor efficacy may be achieved with selected analogs of imexon.

Refractory Solid Tumors: In the *in vitro* screening panel, a number of analogs showed roughly equipotent sensitivity across the 12 cell line panel. For several analogs tumor cell sensitivity increased in a few of the non-hematologic (or solid) tumor cell lines. This was the case for AMP 404, 415 and 416 in all 3 MCF-7 breast cancer cell lines and in the parental WiDr colon cancer cell line. Importantly, this analog has already demonstrated antitumor activity in multidrug-resistant 8226 myeloma cells growing in SCID mice. The consistent observation of maintained potency in cell lines selected for multidrug resistance to natural products further suggests that the analogs could be used in salvage therapy regimens for solid tumor patients who relapse or fail to respond after initial existing chemotherapy agents. Of note, this was the type of official indication recently given for the FDA approval of taxotere in patients with advanced breast cancer who relapse after receiving therapy with regimens containing the natural product DNA intercalator, doxorubicin. Based on the *in vitro* database, the imexon analogs should have clinical applicability in advanced breast cancer, lung cancer and possibly in colon cancer.

The prior preliminary clinical studies with imexon buttress the notion that imexon analogs may be useful in drug-refractory solid tumors. In an early report of European Phase I studies with imexon in cancer patients with a variety of advanced solid tumors, imexon induced objective (measurable) responses in 1/7 evaluable patients each with lung cancer, melanoma and breast cancer. (Mickshe, *et al., Cancer Treat Symp* 1:27-35, 1985.) Notably, the responses in lung cancer and melanoma involved complete disappearance of all measurable

disease for 33 and 42 months, respectively. Furthermore, there were 8 additional patients with advanced tumors who experienced disease stabilization with durations ranging from 4-24 months. This is remarkable since all of these patients were heavily pretreated with drugs and/or radiotherapy, and by definition, in Phase I study, the optimal drug dose is not being used. A more recent report from this group has confirmed these initial observations of imexon activity for imexon doses of 1,200 mg/4 weeks up to 11,000 mg over a 24-month period. (Sagaster, *et al.*, *J Natl Cancer Inst* 87(12):935-936, 1995.) In the one patient with metastatic non-small cell lung cancer (NSCLC), a complete remission has been documented for over 14 years, and stable disease was achieved in 6 patients: one with NSCLC, four with breast cancer and one with liver cancer. (Sagaster, *et al.*, *J Natl Cancer Inst* 87(12):935-936, 1995.) The only consistent toxic effect noted in these patients was mild, transient nausea (13%) and vomiting (23%). Thus, imexon appears to have activity in drug-refractory solid tumors at doses which produce only mild short-term toxic effects.

EXAMPLE 23

Clinical Applications

A. Treating myeloma.

The following protocol is appropriate for Analogue 404 to treat human myeloma which is a B-lymphocyte-derived bone marrow disease. Analogue 404 is formulated in a sterile, isotonic standard saline or dextrose buffer at pH 7.0 at a concentration of 500 μg/ml. The solution is administered intravenously to a patient with hematologic cancer at a rate of 5,000 μg/minute duration via an intravenous route. The total dose is 3.5 g/m^2 given 500 mg/m^2/day x 7 days as a continuous IV infusion.

Anti-tumor effects can be measured using the level of circulating myeloma M protein and bone marrow biopsy. Other therapeutic effects which are observed are an improvement of anemia, a correction of hypercalcemia and a diminution of bone pain. Treatment is repeated every two to four weeks until the patient responds in a suitable manner.

B. Treatment of Melanoma.

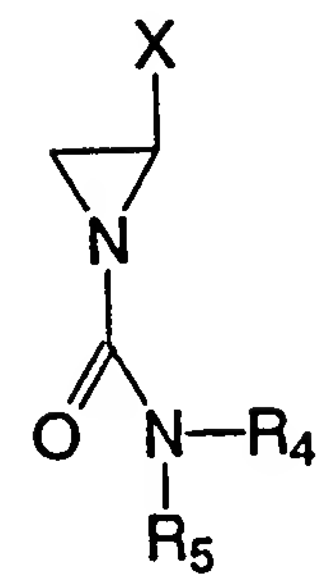
Using the treatment protocol for example 23, but substituting a patient suffering from melanoma. Patient metastases are monitored every 8-12 weeks by x-rays and scans.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A compound of the formula 1:



wherein X is CN, CO_2R_1 or $CONR_2R_3$

where R_1 is an alkyl of 1-6 carbons, a cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons or a lower alkyl substituted aryl of 7-12 carbons;

R_2 is hydrogen or lower alkyl of 1-4 carbons, and

R_3 is hydrogen or lower alkyl of 1-4 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons or heterocyclic ring of 4-16 ring members;

wherein R_4 is hydrogen or lower alkyl of 1-4 carbons; and,

wherein R_5 is an alkyl of 1-8 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 1-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons, a heterocyclic group of 4-16 members, with the proviso that when X is CN, and R_4 is hydrogen, then R_5 is not CH_3, C_6H_5, or, p-nitrophenyl.

2. A compound of claim 1:

wherein X is CN, CO_2R_1 or $CONR_2R_3$

where R_1 is an alkyl of 1-6 carbons, a cycloaklyl of 4-7 carbons, alkenyl of 3-6 carbons or an lower alkyl substituted aryl of 7-12 carbons;

where R_2 is hydrogen or lower alkyl of 1-4 carbons,

where R_3 is a lower alkyl of 1-4 carbons, a lower cycloalkyl of 4-7 carbons, an alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a heterocyclic ring of

4-16 members or a substituted aryl or substituted heterocyclic ring where said substitutents are 1 or 2 and independently selected from the group consisting of lower alkyl of 1-4 carbons, nitro, halo substituted lower alkyls of 1-4 carbons, a lower alkyl substituted acyloxy of 1-5 carbons, a lower alkyl substituted acyl of 1-5 carbons; and,

where R_2, R_3 and N may be taken together to form a heterocyclic ring of 4-16 ring members; and

wherein R_4 is hydrogen or lower alkyl of 1-4 carbons; and,

wherein R_5 is an alkyl of 1-8 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons having 1-2 substitutents wherein the substituents are independently selected from the group consisting of lower alkyl of 1-4 carbons, nitro, halo substituted lower alkyls of 1-4 carbons, a lower alkyl substituted acyloxy of 1-5 carbons, a lower alkyl substituted acyl of 1-5 carbons, a heterocyclic group of 4-16 members.

3. A compound of claim 1 wherein X is CN.

4. A compound of claim 2 wherein

R_4 is hydrogen; and

R_5 is a straight chain alkyl of 1 to 8 carbons, an unsubstituted aryl, a mono-substituted or disubstituted aryl wherein the aryl is independently substituted with halo, lower alkyl, halo substituted lower alkyl, acyl or lower alkyl-substituted acyloxy.

5. A compound of claim2 wherein R_4 is hydrogen; and R_5 is a heterocyclic group or a substituted aryl.

6. A compound of claim 2 wherein R_4 is hydrogen; and R_5 is a pyridyl, a substituted phenyl or a naphthyl.

7. A method of treating cancer, wherein the cancer is selected from the group comprising multiple myeloma, a ß-lymphocyte plasmacytoma,

advanced stage ovarian epithelial cell cancer, metastatic melanoma, leukemias of lymphoid and nonlymphoid origin, metastatic colon cancer, breast cancers and metastatic lung cancers, by administering to a patient in need of treatment an a unit dose of a compound of formula 1:

wherein X is CN, CO_2R_1 or $CONR_2R_3$

where R_1 is an alkyl of 1-6 carbons, a cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons or a lower alkyl substituted aryl of 7-12 carbons,

R_2 is hydrogen or lower alkyl of 1-4 carbons, and

R_3 is lower alkyl of 1-4 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 3-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons or heterocyclic ring of 4-16 ring members;

wherein R_4 is hydrogen or lower alkyl of 1-4 carbons; and,

wherein R_5 is an alkyl of 1-8 carbons, lower cycloalkyl of 4-7 carbons, alkenyl of 1-6 carbons, an aryl of 4-10 carbons, a substituted aryl of 4-12 carbons, a heterocyclic group of 4-16 members; and,

wherein said unit dose is effective to reduce at least one of the symptoms of the cancer.

8. A method of claim 6 wherein X is cyano.

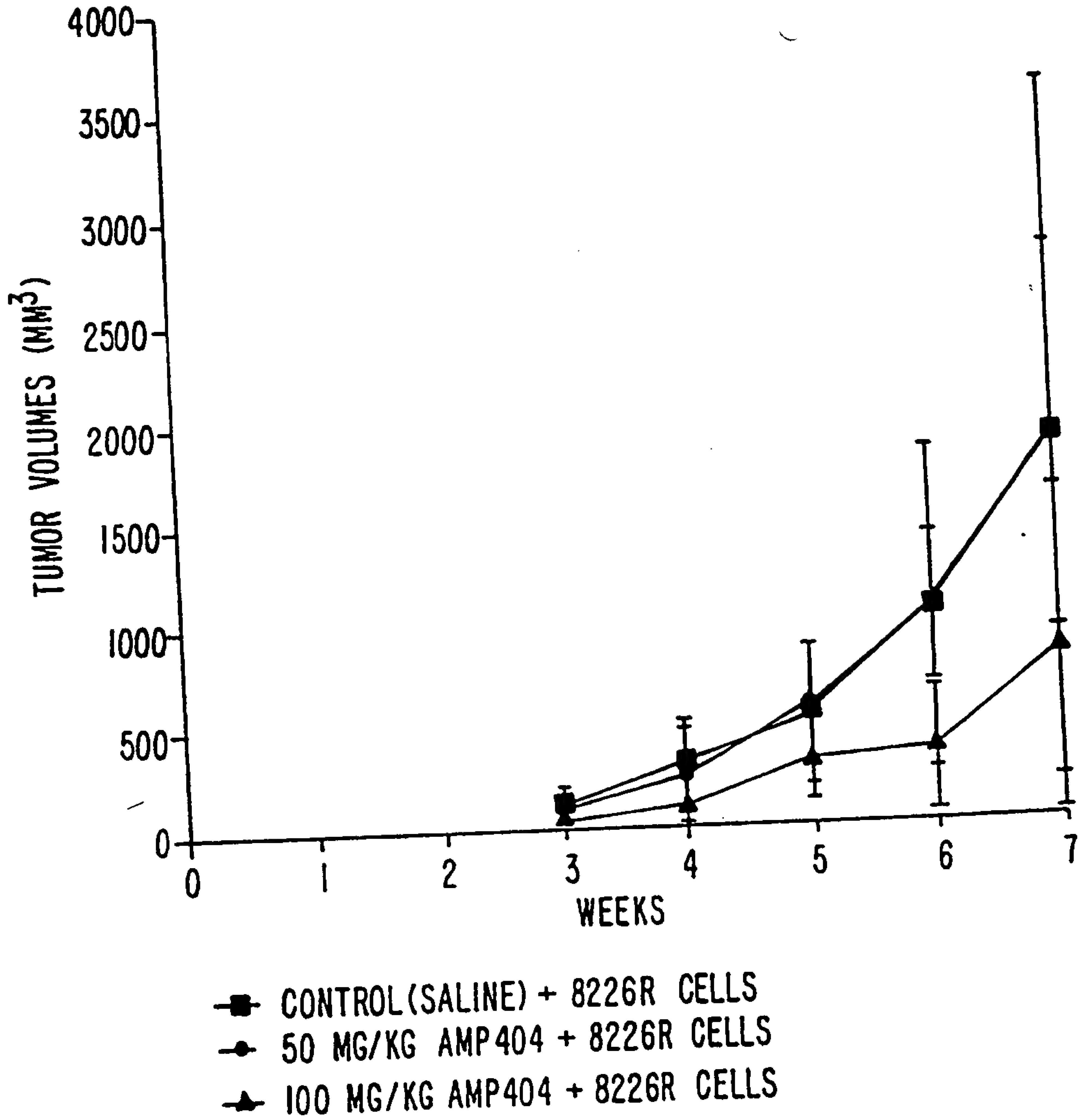
9. A method claim 6 wherein the unit dose is 0.25 to 2 grams.

10. A method of claim 6 wherein the unit dose is administered via a parenteral route.

11. A method of Claim 6 wherein the cancer is selected from the group comprising multiple myeloma, a ß-lymphocyte plasmacytoma, advanced stage ovarian epithelial cell cancer, metastatic melanoma, leukemias of lymphoid and nonlymphoid origin, metastatic colon cancer, breast cancers and metastatic lung cancers.

12. A pharmaceutical composition comprising a unit dose of a compound of claim 1 in a sterile aqueous solution, or in a water-miscible formulation.

13. A pharmaceutical composition of claim 11 which further comprises pharmaceutically acceptable excipients.



4000
3500
3000
2500
2000
1500
1000
500
0
TUMOR VOLUMES (MM3)
0
1
2
3
4
5
6
7
WEEKS
CONTROL(SALINE) + 8226R CELLS
50 MG/KG AMP404 + 8226R CELLS
100 MG/KG AMP404 + 8226R CELLS

FIG. 1.

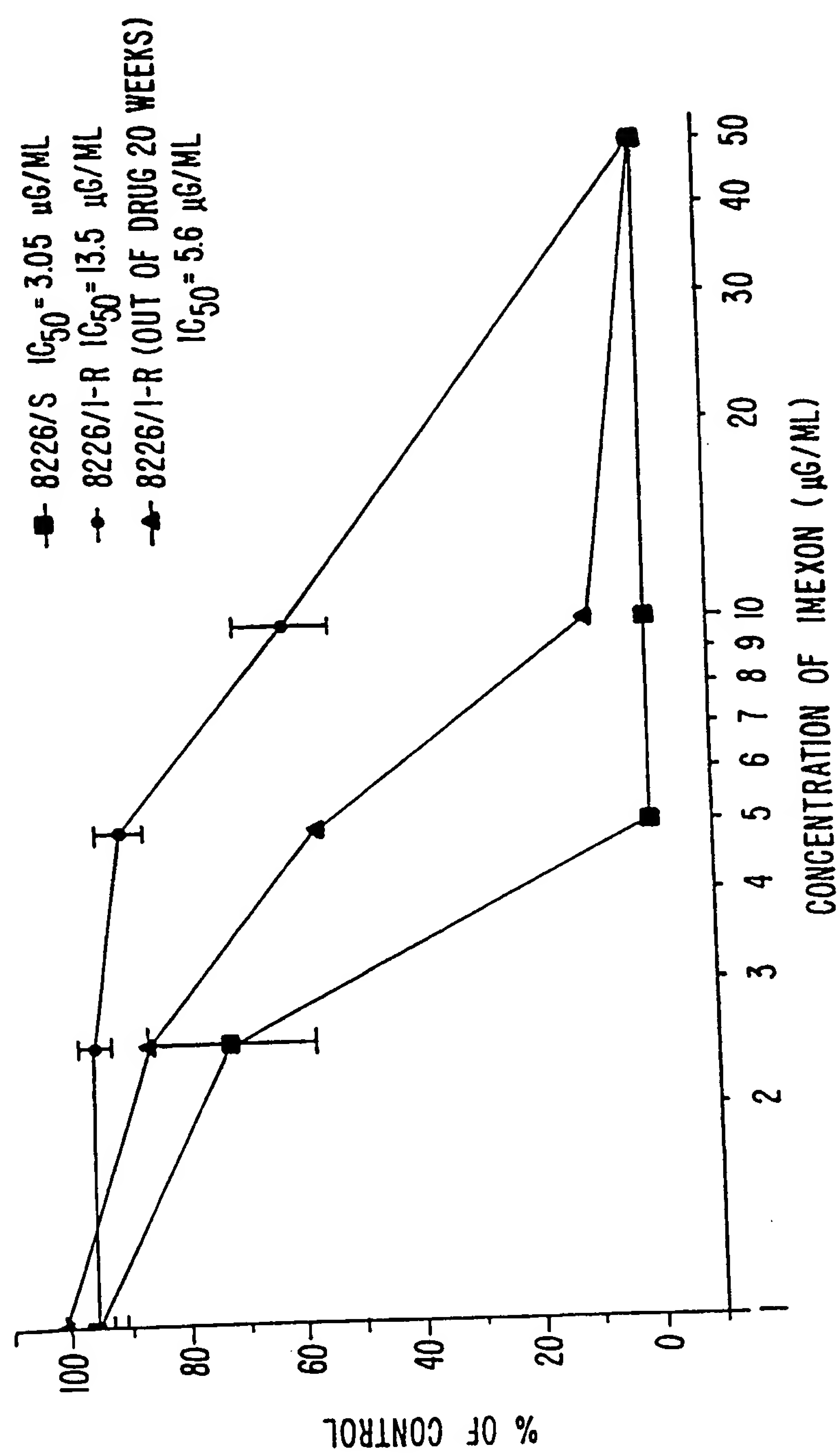
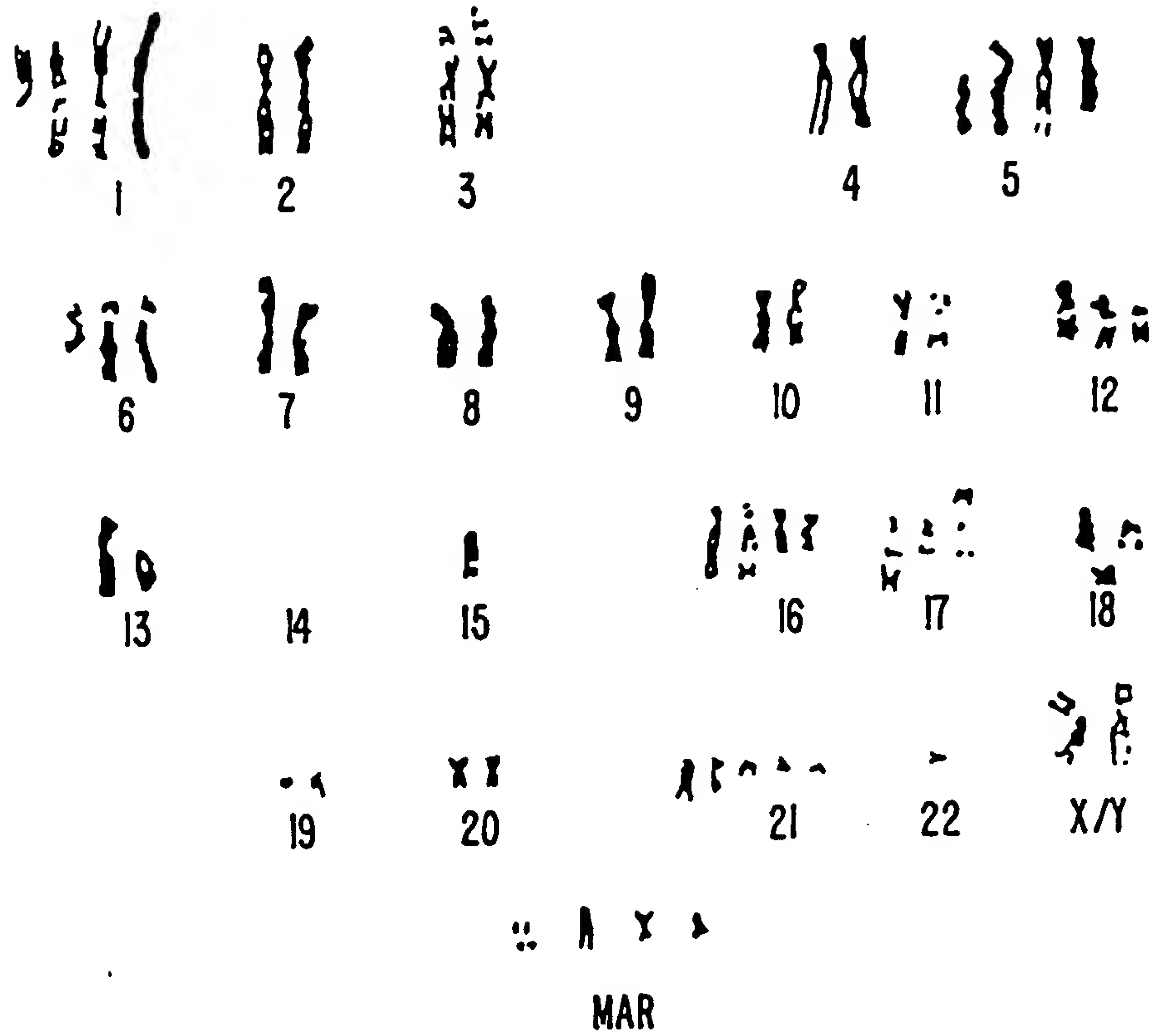

8226/S IC50 = 3.05 μG/ML
8226/I-R IC50 = 13.5 μG/ML
8226/I-R (OUT OF DRUG 20 WEEKS) IC50 = 5.6 μG/ML
% OF CONTROL
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50
CONCENTRATION OF IMEXON (μG/ML)

FIG. 2.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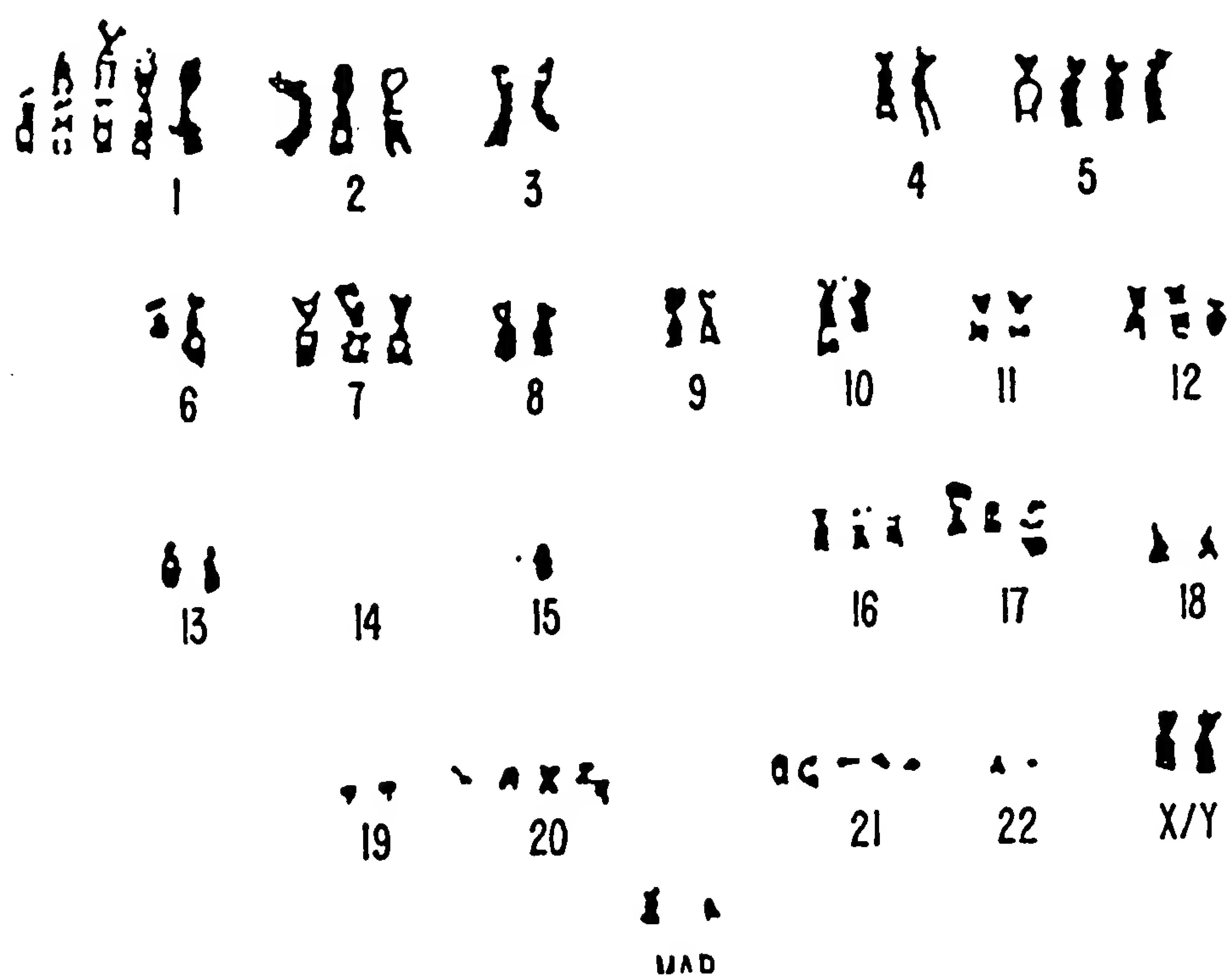
X/Y
MAR

FIG. 3A.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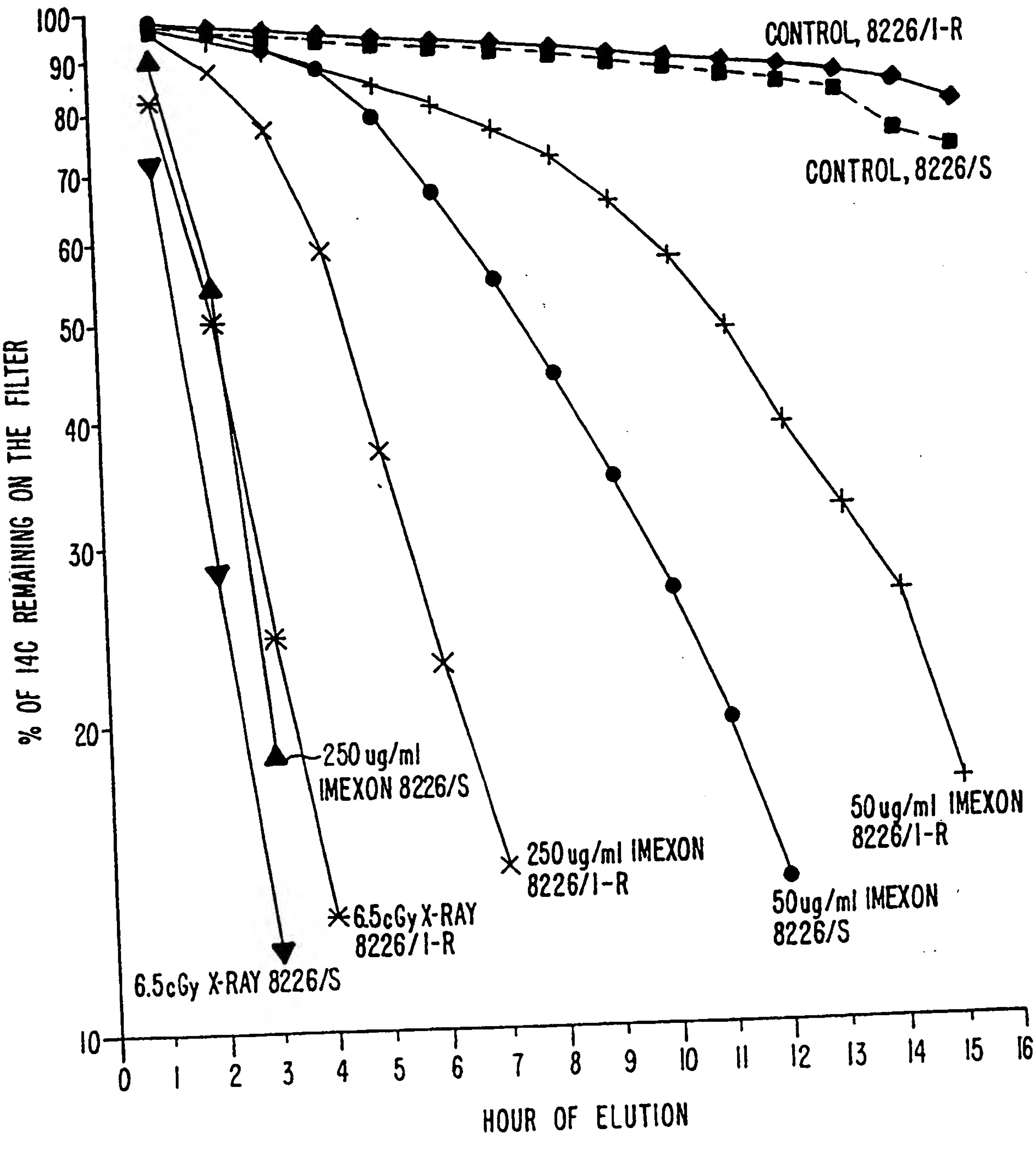
21
22
X/Y

FIG. 3



100
90
80
70
60
50
40
30
20
10
% OF 14C REMAINING ON THE FILTER
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
HOUR OF ELUTION
CONTROL, 8226/I-R
CONTROL, 8226/S
250 ug/ml IMEXON 8226/S
250 ug/ml IMEXON 8226/I-R
50 ug/ml IMEXON 8226/I-R
50 ug/ml IMEXON 8226/S
6.5cGy X-RAY 8226/I-R
6.5cGy X-RAY 8226/S

FIG. 4.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13346

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/165, 31/395, 31/44; C07D 203/16, 401/02, 401/06
US CL :514/183, 340, 624; 546/268.1; 548/962, 966; 562/190
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/183, 340, 624; 546/268.1; 548/962, 966; 562/190

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

HACHK'S CHEMICAL DICTIONARY

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE
aziridine?, carboxamid?, tumor, cancer, oncolog?, melanoma, leukemia, myeloma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,517,183 A (BOSIES et al) 14 May 1985, entire document.	1-13
Y	US 4,282,212 A (BERGER et al) 04 August 1981, see entire document, especially abstract.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 04 AUGUST 1998	Date of mailing of the international search report 08 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JANE C. OSWECKI JOB for Telephone No. (703)308-1235

EXHIBIT 7

US005435989A

United States Patent [19]

Presant et al.

[11] **Patent Number: 5,435,989**

[45] **Date of Patent: * Jul. 25, 1995**

[54] **METHOD OF TARGETING A SPECIFIC LOCATION IN A BODY**

[75] Inventors: **Cary A. Presant**, San Marino; **Richard T. Proffitt**, Arcadia; **Raymond L. Teplitz**, Pasadena; **Lawrence E. Williams**, San Dimas; **George W. Tin**, Arcadia, all of Calif.

[73] Assignee: **Vestar, Inc.**, San Dimas, Calif.

[*] Notice: The portion of the term of this patent subsequent to May 28, 2008 has been disclaimed.

[21] Appl. No.: **663,503**

[22] Filed: **Oct. 22, 1984**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 363,593, Mar. 30, 1982, abandoned.

[51] **Int. Cl.**[6] **A61K 51/12**; A61K 9/133

[52] **U.S. Cl.** **424/1.21**; 424/450; 600/3; 436/829

[58] **Field of Search** 424/1.1, 450, 1.21, 424/1.45; 436/829; 600/3

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[57] **ABSTRACT**

Micellular particles such as small unilamellar vesicles of less than 2000 Å loaded with ^{111}In are administered to BALB/c mice in which EMT6 tumors had been induced. Whole body scintographs of the mice to which either neutral or positively or negatively charged vesicles had been administered show a substantial quantity of the vesicle entrapped ^{111}In localized in the tumor. Blocking of macrophages in the liver and spleen by first administering unlabeled, aminomannose substituted vesicles before administration of the labeled vesicles increases uptake of the ^{111}In labeled vesicles in the tumor.

35 Claims, 3 Drawing Sheets

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Tissue	% injected dose per per gram of tissue*
Blood	1.1 ± 0.2
Tibias	5.5 ± 0.5
Lung	3.7 ± 0.8
Liver	50.7 ± 2.1
S & L Intestine#	0.9 ± 0.0
Kidney	5.9 ± 0.3
Spleen	73.2 ± 13.9
Carcass	0.9 ± 0.1
Stomach#	0.2 ± 0.0
Muscle	0.3 ± 0.0
Skin	0.8 ± 0.0
S.C. LLC Tumor	2.1 ± 0.1
Granuloma	0.6 ± 0.1
% of Recovery	81.8 ± 0.9
Tumor Mass (mg)	4 ± 1

*Mean values ± standard error of the mean
N = number of mice per group
# values include organ contents

FIG. 1

24 h % Injected Dose Per Gram of Tissue*

Number of days after subcutaneous implantation of Lewis Lung Carcinoma

Tissue	8 N=4+	11 N=4+	17 N=4+
Blood	8.1 ± 0.01	11.4 ± 2.3	1.3 ± 0.4
Tibias	6.4 ± 0.6	6.8 ± 0.6	4.7 ± 0.7
Lung	5.7 ± 0.5	15.1 ± 1.4	12.6 ± 1.6
Liver	50.0 ± 2.4	50.5 ± 1.9	36.1 ± 3.2
S & L Intestine#	4.3 ± 0.2	4.9 ± 0.6	1.8 ± 0.3
Kidney	15.1 ± 0.5	15.8 ± 0.4	9.2 ± 0.2
Spleen	57.0 ± 5.6	50.0 ± 6.7	22.2 ± 3.2
Carcass	2.3 ± 0.1	2.8 ± 0.2	1.6 ± 0.1
Stomach*	3.5 ± 0.6	4.3 ± 1.3	1.5 ± 0.5
Muscle	1.1 ± 0.1	0.8 ± 0.0	0.4 ± 0.0
Skin	5.6 ± 2.7	2.1 ± 0.3	2.4 ± 0.2
S.C. LLC Tumor	23.7 ± 2.7	17.0 ± 3.3	9.8 ± 0.9
% of Recovery	102.8 ± 0.6	105.0 ± 1.9	91.3 ± 1.5
Tumor Mass (g)	0.13 ± 0.04	0.16 ± 0.04	1.67 ± 0.39

*Mean values ± standard error of the mean
N = number of mice per group
# values include organ contents

FIG.2

TISSUE	DS PC:Ch:AM (8:3:1) (AM/2) BLOCKADE	In ^{111}In-NTA DS PC:Ch (2:1)	In ^{111}In-NTA DS PC:Ch:SA (4:1:1)	In ^{111}In-NTA DS PC:Ch:DP (4:1:1)
TUMOR	+	26.4	11.8	11.7
	-	18.5	6.1	11.9
LIVER	+	10.2	17.6	17.3
	-	14.6	28.5	16.6
SPLEEN	+	10.5	32.4	32.7
	-	18.8	43.8	39.3
LUNG	+	8.0	2.8	3.8
	-	6.0	1.8	3.0
KIDNEY	+	6.6	7.8	17.8
	-	6.8	6.8	17.1
BLOOD	+	7.9	2.4	1.7
	-	6.6	1.0	1.3

FIG.3

TREATMENT	[^{3}H] MTX IN TUMOR (dpm/gm)	TREATMENT CONTROL	[^{14}C] LIPID IN TUMOR (dpm/gm)
FREE [^{3}H] MTX	6,700	1.0	–
LIPOSOME ENTRAPPED [^{3}H] MTX	20,150	3.0	12,570
LIPOSOME ENTRAPPED [^{3}H] MTX; AFTER AM/2 BLOCKADE	19,000	2.8	12,670

FIG.4

METHOD OF TARGETING A SPECIFIC LOCATION IN A BODY

BACKGROUND OF THE INVENTION

RELATIONSHIP TO OTHER APPLICATIONS

This application is a continuation-in-part of application, Ser. No. 363,593, filed Mar. 30, 1982, now abandoned "Method of Targeting a Specific Location in a Body".

FIELD OF THE INVENTION

This invention relates to a method of targeting specific locations as, for example, tumors, in a body, by use of micellular particles such as phospholipid vesicles. The invention may be used for diagnosis and/or treatment of such abnormalities.

DESCRIPTION OF PRIOR ART

Before various abnormalities in a patient's body can be diagnosed and treated, it is often necessary to locate the abnormalities. This is particularly true of abnormalities such as malignant tumors since the treatment of such tumors is often on a localized basis. For example, the location of cancer cells has to be identified so that a therapeutic agent can be directed to such cells to eliminate the tumor.

Various attempts have been made over an extended number of years to identify specific locations, such as tumors, in a patient's body by simple techniques. For example, it would be desirable to identify the location of cancer cells by a simple method involving the introduction of a particular chemical to the patient's body and the movement of such chemical to such specific locations. It would also be desirable to treat the cancer by introducing modified chemicals into the patient's body and having such chemicals move to specific locations to combat the cancer cells at such locations. In spite of such attempts, however, simple delivery systems for targeting specific locations, such as tumors, for treatment or diagnosis do not exist as yet.

Placing a chemotherapeutic drug in the body orally, subcutaneously or intravenously can result in harm to the normal cells in the body which take up the drug and a worsening in the patient's condition, without achieving the desired reduction in tumor cell activity. In the past, this toxicity to normal cells in the patient's body has been a major disadvantage in the treatment of tumors with chemotherapeutic agents. The lack of efficacy of such chemotherapy is also attributable to the failure of the freely circulating drug to localize within tumor cells before it is excreted or taken up by other cells in the body.

Prior attempts to improve treatment of tumors by chemotherapeutic agents have included encapsulation of such agents within biodegradable phospholipid micellular particles in the form of vesicles or liposomes. Encapsulation is thought to reduce the potential toxicity from the circulating drugs. Researchers have also sought to utilize such encapsulation to selectively target tumors within a body for delivery of chemotherapeutics. However, until the invention disclosed in the present application and the related application Ser. No. 363,593, efforts to locate or treat tumor cells with drug-encapsulating targeting particles have not been successful.

The inability to provide a satisfactory targeting method is believed to be due to the nature of the solid tumors and their metastases which are located in extravascular tissues. Thus, to accomplish targeting of intravenously injected radiolabelled or chemotherapeutic agents to the tumor cells, the agents must leave the normal circulation by crossing the blood vessel membranes to enter the extravascular tissues. This movement is known as "extravasation". In addition the encapsulated agent must cross the tumor cell membrane. Normally, small substances such as small molecular weight proteins and membrane-soluble molecules can cross cell membranes by a process known as passive diffusion. However, passive diffusion will not allow sufficient accumulation of larger particles carrying drugs within cells to reach therapeutic levels. Additionally, cells can actively transport materials across the membrane by a process such as pinocytosis wherein extracellular particles are engulfed by the membrane and released inside the cell. Entry of encapsulating particles into individual cells may occur by pinocytosis.

Progress in targeting such specific locations with chemotherapeutic drugs has been hampered by the inability to accomplish and detect movement of drug carriers across blood vessel membranes. In the usual case, large structures such as drug encapsulating vesicles cannot escape from blood vessels such as capillaries, and thus remain in circulation.

An understanding of extravasation, however, requires an examination of the structure of the vascular morphology of a tumor. Various blood vessels are associated with tumors, in particular capillaries. It is now known that tumor capillaries may exhibit alterations in their structure, such as fenestrations, as a result of tumor cell growth patterns. H.I. Peterson, *Vascular and Extravascular Spaces in Tumors: Tumor Vascular Permeability*, Chapter III, Tumor Blood Circulation, H. I. Peterson, Ed. (1979). Studies of tumor capillary permeability reveal morphologic variations in the capillaries which allow some substances to cross the capillary membrane. Such variations include defects in vascular endothelium from poor cell differentiation, or breaks in vascular walls as a result of invading tumor cells. H.I. Peterson, supra.

Notwithstanding such knowledge of tumor vascular morphology, researchers such as Peterson have concluded that transport of large molecules or materials across the tumor capillary wall occurs as a result of passive diffusion and that "concentrations of active drugs sufficient for therapeutic effect are difficult to reach." H. I. Peterson, supra, at 83.

Prior to such morphologic studies, early reports suggested that vesicles might undergo transcapillary passage across the capillary membranes into tumor cells. G. Gregoriadis, *Liposomes in Biological Systems*, Gregoriadis, Ed., Ch 2, (1980). However, available data indicated that the vesicles were unstable in vivo and that the radiolabel may have leaked, thus apparently prompting alternative theories such as longer circulation of vesicles in the blood with release of drugs at a slower rate and interaction of the liposomes with the capillary walls without crossing the wall surface, which presumably resulted in the drugs within tumors. Id. Other researchers simply have concluded that the vesicles do not penetrate vascular walls after intravenous administration. B. Ryman et al., *Biol. Cell*, Vol 47, pp. 71–80 (1983); G. Poste, *Biol. Cell*, Vol. 47, pp. 19–38 (1983).

Thus, although the prior art has recognized that vesicles carrying therapeutic drugs must cross vascular barriers to reach tumor cells, the experience of the art has taught that intravenous administration is not effective to deliver encapsulated drugs to extravascular tumor cells. This invention accordingly provides simple methods of enhancing extravasation of encapsulated chemotherapeutic agents to tumor cells within a body. The method of this invention further provides for the identification of such tumor sites in the body.

SUMMARY OF THE INVENTION

The method of this invention includes the provision of phospholipid micellular particles such as vesicles. Pure (more than approximately 98% pure) neutral phospholipid molecules are incorporated into small (less than 2000Å) micelles so that they are a component of external surface. The phospholipid molecules and/or vesicle contents may be radiolabeled to enhance the identity of the specific location and the diagnosis of the tumor at the specific location.

The phospholipid molecules may constitute distearoyl phosphatidylcholine. The stability of the distearoyl phosphatidylcholine micelles may be enhanced by the incorporation of cholesterol. Positively charged molecules such as stearylamine or aminomannose or aminomannitol derivatives of cholesterol or negatively charged molecules such as dicetyl phosphate may also be incorporated into the vesicles.

When phospholipid micelles are introduced into the blood stream of a patient, the micelles move to the specific locations of cancerous growth in the patient's body, which may then be identified and treated. Drugs may be included in phospholipid vesicles and such drug-bearing vesicles may then be introduced into the patient's body for targeting the tumor locations.

To enhance movement of the phospholipid vesicles to the specific locations, positively charged phospholipid vesicles may first be introduced into the patient's blood stream to block the macrophages in the patient's body. The positively charged molecules bound to such phospholipid vesicles may be an aminomannose or aminomannitol derivative of cholesterol. Concurrently or after a suitable period of time such as approximately one (1) hour, other phospholipid vesicles may be introduced into the patient's blood stream to move to the specific locations in the body. Such phospholipid vesicles may include cholesterol and may be neutral or may be positively charged as by the inclusion of a stearylamine or aminomannose or aminomannitol derivative of cholesterol or may be negatively charged as by the inclusion of a dicetyl phosphate.

When the phospholipid vesicles are introduced into the body to target tumors, indium-111 may be used as the labelling agent. The indium-111 may be chelated to a suitable material such as nitrilotriacetic acid (NTA). NTA is advantageous because it forms a weak bond with the indium-111. As a result, when the phospholipid vesicles reach the tumor, the nitrilotriacetic acid is displaced by proteins at the tumor. Since the proteins have a strong bond with the indium-111, the indium-111 remains at the tumor for a long period of time (in excess of 24 hours), which provides for easy identification of the tumor over the extended period of time.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a table illustrating the targeting of phospholipid vesicles to tumors in a body;

FIG. 2 is a table illustrating the targeting and blocking of macrophages in the liver and spleen by phospholipid vesicles;

FIG. 3 is a table illustrating the targeting of phospholipid vesicles to tumors in the body after the blocking of the macrophages in the liver and spleen; and

FIG. 4 is a table illustrating the enhanced delivery of drugs to a tumor in a body by the use of phospholipid vesicles.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "micellular particle" and "micelles" refer to particles which result from aggregations of amphiphilic molecules. In this invention preferred amphiphiles are biological lipids. Micelles are water-soluble aggregates of molecules with hydrophobic and hydrophilic portions (so-called amphiphilic molecules) which associate spontaneously. Such micelles can be in the form of small spheres, ellipsoids or long cylinders, and can also consist of bilayers with two parallel layers of amphiphilic molecules. Such bilayered micelles usually take the shape of spherical vesicles with an internal aqueous compartment. Useful compositions of these micelles include phospholipid molecules in the structure.

"Vesicle" refers to a micelle which is in a generally spherical form, often obtained from a lipid which forms a bilayered membrane and is referred to as a "liposome". Methods for forming these vesicles are, by now, well known in the art. Typically, they are prepared from a phospholipid, for example, distearoyl phosphatidycholine, and may include other materials such as neutral lipids, for example, cholesterol, and also surface modifiers such as positively or negatively charged compounds.

Materials and Methods

Liposome Preparation. Small unilamellar vesicles (SUV) with the ionophore A23187 were prepared from distearoyl phosphatidycholine (DSPC), cholesterol (Ch), dicetyl phosphate (DP), stearylamine (SA) and the 6-aminomannose (AM), and 6-aminomannitol (AML) derivatives of cholesterol, according to previous methods. Briefly, chloroform solutions of 10 mg lipid with the following molar ratios: DSPC:Ch, 2:1; DSPC:Ch:X, 4:1:1 where X=SA, DC or AML; and DSPC:Ch:AM, 8:3:1, were evaporated to dryness under nitrogen (N_2,) and further dried under vacuum overnight. Each tube was filled with 0.6 ml phosphate 10 mM phosphate buffered 0.9 saline, pH 7.4(PBS), containing 1 mM nitrilotriacetic acid (NTA) and sonicated under N_2, for 5 to 15 minutes with a MSE sonicator equipped with a titanium microtip.

Liposomes were annealed at 60° C. for 10 minutes and centrifuged at 300×g. Liposomes were separated from unencapsulated NTA with a 30×1.5 cm Sephadex G-50 column. Liposome size was determined by electron microscopy of preparations negatively stained with uranyl acetate. All vesicle types were shown by electron microscopy to have a mean diameter less than 0.1 microns (1000Å). For example, DSPC:Ch vesicles had a mean diameter of approximately 528Å. However, vesicles as large as approximately 2000 Angstroms are believed to be satisfactory in obtaining the desired results of this invention, although the preferred range is approximately 500 to about 700Å.

The vesicles obtained as described above are chemically pure. By "chemically pure" is meant that the materials which constitute phospholipid vesicles are more than 98% pure. For example, when the phospholipid chemical added is distearoyl phosphatidylcholine, this material is used at more than 98% purity. The same constraint holds for other components, such as cholesterol, which compose the vesicle. The phospholipid vesicles obtained as described above are stable when injected into experimental animals.

The aminomannose and aminomannitol derivatives of cholesterol extend externally from the phospholipid particles. Thus, when such derivatives are incorporated or associated into the surfaces of vesicles or other micelles, an amine moiety is provided that extends approximately 5–15 Angstroms, preferably about 10 Angstroms, beyond the surface of the micelles. In the case of vesicles, it appears that the appropriate molecular design comprises a hydrophobic portion which serves to anchor the molecule within the vesicular bilayer, and a linking portion which is at least mildly hydrophilic which spans the requisite distance between the hydrophobic region and the amino functional group. The hydrophilicity is apparently required to prevent the link from internalizing within the bilayer also and thus serves to "extend" the amine from the surface. An example of a successful extended amine within the context of this invention is a 6-aminomannose cholesterol derivative such as, for example, 6-(5-cholesten-3β-yloxy)hexyl-6-amino-6-deoxyl-thio-D-mannopyranoside. In this example, the cholesterol portion provides the hydrophobic moiety, while the aminomannose is relatively hydrophilic. Other embodiments are also possible: other amino sugars attached to other cholesterol derivatives, for example, are equally suitable as alternative embodiments of the hydrophilic and hydrophobic portions. Polyamines and polyamino acids which can be bound covalently or associated by other means to the vesicle or other micelle surface may also be used.

The amino derivatives and cholesterol tend to impart stability to the phospholipid vesicles. Cholesterol may be included in the range of approximately 0% to 50% of cholesterol by weight and the remainder constituting the phospholipids. The charged molecules such as the stearylamine, the dicetyl phosphate and the aminomannose and aminomannitol derivatives of cholesterol may be included in the range of 0% to 20% by weight of the charged molecules and the remainder constituting the phospholipids.

The chemically pure liposome compositons discussed above are quite stable to leakage in vitro and in vivo. However, phospholipid mixtures such as egg lecithin form more fluid membranes than pure phospholipids. As a result, liposomes from natural lecithin mixtures are less stable to leakage of their contents than pure phopholipids.

In-111 Loading Procedure. Loading of In-111 into preformed liposomes was facilitated by the presence of A23187 in the lipid bilayer. In-111 was loaded into liposomes at 60°–80° C. as previously described. Incubations were terminated by the addition of 10mM ethylenediaminetetraacetic acid (EDTA) in 10 mM phosphate buffered 0.9% sodium chloride, pH 7.4 (PBS), and free In-111 was separated from the loaded liposomes by chromatography on Sephadex G-50. Up to 90% of the added In-111 could be incorporated into preformed liposomes by this technique, and specific activities of up to 300 μCi/mg lipid have been obtained.

EMT6 Tumor Growth. Male BALB/c mice weighing 20–25 g were injected subcutaneously on the right hind leg with 5×10 EMT6 cells in 0.1 ml sterile phosphate buffered saline. Tumors were allowed to grow for 10–20 days prior to using these animals for imaging studies. At this stage, tumors weighed between 0.2 and 0.4 gm. Up to 0.5 ml PBS containing 1 to 2 mg liposomes loaded with up to 30 μCi in-111 were injected into the tail vein of each animal. Control animals were injected with In-111-NTA which had not been encapsulated in liposomes.

Gamma Camera Imaging. At one (1) hour and at twenty-four (24) hours after injecting In-111 loaded liposomes, each animal was anesthetized with 40 mg/kg sodium pentobarbital and positioned on a platform 12 cm from the gamma scintillation camera equipped with a 6 mm pinhole. Whole-body dorsal images were acquired on x-ray film and corresponding digitized data were stored on magnetic discs for computer analysis.

Biodistribution of Radioactivity. Immediately after the twenty-four (24) hour images were acquired, animals were sacrificed and dissected to determine the organ distribution of radioactivity. Organs or tissues were excised, washed in PBS, blotted dry, and weighed. Radioactivity was measured in a well-type gamma-ray spectrometer and quantitated based on activity present in liposomes before injection. In some experiments, the gamma-ray perturbed angular correlation technique was used to measure the rotational correlation time of the In-111 in individual tissues and thereby assess the proportion of isotope remaining in intact liposomes.

RESULTS

Whole body scintographs were made of tumor bearing mice which had been injected intravenously with In-111 NTA small phospholipid vesicles SUV 24 hr previously. EMT6 tumor images were clearly discernible in animals injected with neutral, negative and positively charged phospholipid vesicles. A comparison of the biodistribution of In-111 NTA delivery by each of these vesicle types can be made from the data presented in FIG. 1. As will be seen from the second column of FIG. 1, neutral phospholipid vesicles provided the best delivery of In-111 to tumor tissue. The specific targeting of the phospholipid vesicles to the tumors in this instance was at least as high as the targeting of the phospholipid vesicles to the liver or spleen, the usual target tissues of liposomes, and was nearly 8 times greater than the specific activity observed at the tumors when free In-111 NTA was injected in vivo. This will be seen from a comparison of the results shown in the first and second columns of FIG. 1. It can also be seen in FIG. 1 that, as liver and spleen uptake of In-111 decreases, the concentration of the phospholipid vesicles remaining in the blood increases. Also the increase in tumor associated radioactivity correlates approximately with the blood level of In-111.

Applicants have previously demonstrated a strong association with EMT6 tumor cells in vitro of liposomes with 6-aminomannose derivatives of cholesterol. Applicants accordingly attempted tumor imaging with phospholipid vesicles of aminomannose derivatives of cholesterol where such vesicles were labeled with in-111. Applicant's observations in this experiment confirmed that the vast majority of In-111 in such phospholipid vesicles ultimately is deposited in the liver and spleen. Tumor images could not be obtained with such phospholipid vesicles as demonstrated in columns 2 and 3 of

FIG. 2 by the low deposition of the phospholipid vesicles in the tumor. The low deposition of the phospholipid vesicles in the tumor may result from the fact that most of such vesicles are taken up by the liver and spleen.

Liposomes with a lower concentration of the 6-AM derivative of cholesterol do not get trapped in the lung, so it seemed reasonable to assume that AM/2 vesicles (third column of FIG. 2) loaded with In-111 might be better tumor imaging agents than the material shown in the second column of FIG. 2. A comparison of the second and third columns of FIG. 2 shows that this was not the case. In fact, the AM/2 vesicles had a very high affinity for the liver and spleen. For example, after a period of 24 hours from the time of injection of the lipid vesicles in the blood stream, the combined radioactivity in the liver and spleen averaged greater than 75% of the total injected dose. This was the highest amount of liver and spleen uptake of vesicles observed of the several lipid composition studies.

Applicants have previously shown that positively charged liposomes were bound to EMT6 cells in vitro to a much greater extent than either neutral or negatively charged liposomes. Applicants accordingly investigated AML derivatives of cholesterol, another synthetic glycolipid derivative with positive charge. These AML liposomes did show a lower affinity for liver and spleen (Column 4 of FIG. 2) and a slightly increased uptake by tumor compared to that provided by AM/2 liposomes (Column 2 of FIG. 2). However, this level of tumor-associated radioactivity was still three to ten times less than observed in she experiment with neutral, positive and negative liposomes as shown in FIG. 1.

In further experiments, applicants injected mice with either a saline solution or with 8 mg AM/2 liposomes. The saline solution provided a control and did not block the macrophages in the liver and spleen in the manner discussed above. This is shown in FIG. 3. The AM/2 liposomes provided a positive charge and were effective in blocking the macrophages in the liver and spleen. This is also shown in FIG. 3. Since the macrophages in the liver and spleen were blocked, any subsequent injection of phospholipid vesicles into the blood stream of the body had an increased opportunity to become targeted to the tumor.

One hour after the injection of the liposomes as discussed in the previous paragraph, 1 mg of the type of liposomes discussed above in relation to FIG. 1 was injected in the mice. These liposomes contained In-111. Twenty-four (24) hours afterwards, mice were sacrificed and dissected to determine biodistribution of In-111.

FIG. 1 indicates the amount of In-111 targeted to the different parts of the body when phospholipid vesicles containing In-111 are introduced into the blood stream without any previous blockade of the macrophages in the liver and spleen. In contrast, FIG. 3 indicates the amount of In-111 targeted to the different parts of the body when phospholipid vesicles containing In-111 are introduced into the blood stream after a previous blockade of the macrophages in the liver and spleen. As will be seen, the amount of the In-111 targeted to the tumor significantly increased in most instances in FIG. 3 for the individual phospholipid vesicles than for the corresponding phospholipid vesicles in FIG. 1. Furthermore, the amount of the In-111 received at the liver and spleen in FIG. 3 is significantly reduced from the amount of the In-111 received at the liver and spleen in FIG. 1.

As will be seen from a comparison of FIGS. 1 and 3, a significant amount of the-phospholipid vesicles is targeted to the tumor even when the macrophages in the liver and spleen are not previously blocked. However, the amount of phospholipid vesicles targeted to the tumor is substantially increased when the macrophages in the liver and spleen are blocked before the phospholipid vesicles to be targeted to the tumor are introduced into the body.

In the experiments discussed above, the phospholipid vesicles to be targeted to the tumor were introduced into the blood stream approximately one (1) hour after the introduction of the phospholipid vesicles into the bloodstream to block the macrophages it, the liver and spleen. It will be appreciated, however, that other time periods may also be used, including time periods considerably shorter than one (1) hour. Since the phospholipid vesicles blocking the liver and spleen are effective for an extended period, the introduction of the phospholipid vesicles to target the tumor may be considered as concurrent with the introduction of the phospholipid vesicles to block the liver and spleen.

As previously described, neutral DSPC:Ch phospholipid vesicles deliver In-111 to EMT6 murine tumors in sufficient quantity to allow definitive localization of tumor by gamma camera imaging. This tumor-associated specific radioactivity (% dose/gram tissue) is equal to levels achieved in liver and spleen, a finding which was not previously observed by others employing liposomes as tumor imaging agents.

There are several improvements in liposome technology employed in the present invention which may explain why better tumor imaging is achieved than has been previously observed by others. One such improvement is that applicants have loaded In-111 into preformed liposomes. By this highly efficient method, specific activities of 200–300 μCi In-lll/mg lipid have been obtained. Another improvement is that applicants have used highly purified phospholipid vesicles as discussed above.

A further improvement has been that In-111 has been encapsulated in the NTA complex. NTA is a relatively weak chelator and, in the presence of serum, NTA is displaced. Thus, when the phopholipid micelles containing the In-111 are targeted to the tumor, the NTA becomes displaced by protein at the tumor. The In-111 becomes tightly associated with the protein at the tumor. Since this protein is within a cell, the In-111 is fixed at the position of the tumor. This circumstance provides two distinct advantages for the purpose of imaging. The first is that little radioactivity is lost due to leakage. After correcting for decay, applicants typically observed that 90% of the initial radioactivity remained in the animal at least twenty-four (24) hours after injection, based on the times required to accumulate a fixed number of counts with gamma counter. A second advantage is that when a label such as In-111 remains fixed at the site of liposome destruction, one can obtain information on rate, as well as total amount, of liposome uptake by the tissue.

Thus, the high tumor specific activities observed in this study are the result of a continuous accumulation of In-111 within the tumor over a twenty-four (24) hour period. By comparison, EDTA contained within stearylamine vesicles forms a strong chelate in comparison to NTA. EDTA is not displaced at the tumor by prote-

ins. Thus, the In-111 will not remain fixed within the cell. For example, when EDTA was chelated to In-111 in a phospholipid vesicle, only 25% of tumor specific activity was achieved, compared to In-111 NTA loaded liposomes.

The phospholipid vesicles may be used to provide an enhanced delivery of drugs or radionuclides to tumors in the body. This may be seen from the results of experiments specified in the table constituting FIG. 4. In these experiments [^{3}H] Methotrexate (MTX) was injected directly into tumor-bearing mice as a control. The amount of the [^{3}H]MTX is directed to the tumors after a period of four (4) hours is illustrated in the row designated in FIG. 4 as "Free [^{3}H]MTX".

Phospholipid vesicles containing DSPC:Ch:SA in the ratio of 4:1:1 were labeled with [^{14}C] cholesteryl oleate and the [^{3}H]MTX was entrapped in the phospholipid vesicles. As will be seen, the amount of the phospholipid vesicles targeted to the tumors is almost three (3) times greater than the amount of the free MTX directed to the tumor.

The liver and spleen were also blocked in the manner described above and shown in FIG. 2 before the phospholipid vesicles containing DSPC:Ch:SA, as described in the previous paragraph, were targeted to the tumors. The last column of the table in FIG. 4 illustrates the targeting of these phospholipid vesicles to the tumors after the blocking of the liver and spleen. As will be seen, the amount of the phospholipid vesicles targeted to the tumors under such circumstances was almost the same as the amount discussed in the previous paragraph.

As indicated previously, the micellular particles are to be less than 2000Å in diameter, preferably in the 500 to approximately 700Å range. To demonstrate further the significance of such size limitation, Table I shows biodistribution data from four mice that received REV vesicles containing In-111 NTA. The larger vesicles were prepared by reversed phase evaporation following the method of Syoka and Papahadjopoulos, *Proc. Natl. Acad.* Sci. USA, 75: 4194-4198 (1978), and were approximately five times larger than SUV's, but with identical chemical composition. To maximize tumor accumulation of the radiolabel agent, the animals were sacrificed when the mean lesion size was only 4±1 mg. Such structures, approximately 2 millimeters in largest dimension, were within the limits of the dissection technique. Uptake of REV's by the malignancies was only 2.1±0.1% ID/g. This value was somewhat above blood levels, but one tenth or less than that found in comparably sized Lewis Lung Carcinomas using SUV's (Table 2). In order to establish that tumor size effects are not different for the larger vesicles, two additional mice were injected with REV's when their carcinomas were considerably larger. Tumor accumulations were 1.5 and 1.2% ID/g in 320 and 2.10 mg lesions respectively. Thus, larger vesicles of DSPC:Chol have very significantly reduced accumulation in Lewis Lung Carcinoma ($p<0.005$ using Tables 1 and 2).

It has also been found that tumor uptake of encapsulated agent decreases significantly with increasing tumor size. For LLC lesions between 0.1 and 0.5 g, uptake varied between 25 and 12% ID/g. Very small metastases (4 and 8 mg) found in the lung after s.c. implantation had uptake of approximately 50% ID/g. For larger lesions, a slow decrease in tumor accumulation was observed out to 1.8 g where the value approached some 10% ID/g. Most of the variation with size occurred in the range 0.02 to 0.2 g; i.e. between 0.1 and 1.0% of the animal's total mass. It was observed during dissection that the larger tumors had relatively enhanced necrotic zones, which explains, at least in part, this dependence upon size.

Although this invention has been described and illustrated with reference to particular applications, the principles involved are susceptible of numerous other applications which will be apparent to persons skilled in the art. The invention is, therefore, to be limited only as indicated by the scope of the appended claims.

TABLE 1

BIODISTRIBUTION OF REVERSED PHASE EVAPORATION VESICLES (REV) IN MICE WITH LEWIS LUNG CARCINOMA AND GRANULOMA

Tissue	% injected dose per per gram of tissue*
Blood	1.1 ± 0.2
Tibias	5.5 ± 0.5
Lung	3.7 ± 0.8
Liver	50.7 ± 2.1
S & L Intestine#	0.9 ± 0.0
Kidney	5.9 ± 0.3
Spleen	73.2 ± 13.9
Carcass	0.9 ± 0.1
Stomach#	0.2 ± 0.0
Muscle	0.3 ± 0.0
Skin	0.8 ± 0.0
S.C. LLC Tumor	2.1 ± 0.1
Granuloma	0.6 ± 0.1
% of Recovery	81.8 ± 0.9
Tumor Mass (mg)	4 ± 1

*Mean values ± standard error of the mean.
N = number of mice per group
# values include organ contents

TABLE 2

TISSUE DISTRIBUTION OF VESICLE-ENCAPSULATED IN-111-NTA IN MICE WITH SUBCUTANEOUSLY IMPLANTED LEWIS LUNG CARCINOMA

	24 h % Injected Dose Per Gram of Tissue* Number of days after subcutaneous implantation of Lewis Lung Carcinoma		
Tissue	8 N = 4+	11 N = 4+	17 N = 4+
Blood	8.1 ± 0.1	11.4 ± 2.3	1.3 ± 0.4
Tibias	6.4 ± 0.6	6.8 ± 0.6	4.7 ± 0.7
Lung	5.7 ± 0.5	15.1 ± 1.4	12.6 ± 1.6
Liver	50.0 ± 2.4	50.5 ± 1.9	36.1 ± 3.2
S & L Intestine#	4.3 ± 0.2	4.9 ± 0.6	1.8 ± 0.3
Kidney	15.1 ± 0.5	15.8 ± 0.4	9.2 ± 0.2
Spleen	57.0 ± 5.6	50.0 ± 6.7	22.2 ± 3.2
Carcass	2.3 ± 0.1	2.8 ± 0.2	1.6 ± 0.1
Stomach*	3.5 ± 0.6	4.3 ± 1.3	1.5 ± 0.5
Muscle	1.1 ± 0.1	0.8 ± 0.0	0.4 ± 0.0
Skin	5.6 ± 2.7	2.1 ± 0.3	2.4 ± 0.2
s.c. LLC Tumor	23.7 ± 2.7	17.0 ± 3.3	9.8 ± 0.9
% Recovery	102.8 ± 0.6	105.0 ± 1.9	91.3 ± 1.5
Tumor mass (g)	0.13 ± 0.04	0.16 ± 0.04	1.67 ± 0.39

*Mean values ± standard error of the mean
+N = number of mice per group.
Values include organ contents

We claim:

1. A method of targeting a tumor in a body with an agent for the diagnosis or treatment therefor comprising
a) providing micellular particles of less than 2000 Å comprising chemically pure phospholipid molecules;
b) incorporating the agent for diagnosis or treatment into the particles; and

c) introducing the micellular particles into the bloodstream of the body to obtain movement of the particles to the tumor.

2. A process according to claim 1 wherein the agent is a radioactive element.

3. A process according to claim 2 wherein the agent emits gamma radiation.

4. A process according to claim 3 wherein the agent comprises ^{111}In.

5. A method as set forth in claim 1 wherein the particles constitute distearoyl phosphatidylcholine.

6. A method as set forth in claim 5 wherein cholesterol is included in the phospholipid particles to enhance the stability of the particles.

7. A method set forth in claim 1 wherein charged molecules are also attached to the vesicles.

8. A method as set forth in claim 7 wherein the small, chemically pure phospholipid vesicles are neutral and wherein the charged molecules are positively charged or negatively charged.

9. A method of targeting a tumor in a body with an agent for the treatment or diagnosis therefor, comprising:

a) providing small vesicles of less than 2000 Å comprising chemically pure phospholipid vesicles;
b) binding cholesterol to such phospholipid vesicles
c) binding charged molecules to such phospholipid vesicles in the range of approximately 0% to 20% by weight of the charged molecules and the remainder constituting the phospholipids;
d) incorporating the agent for diagnosis or treatment into the vesicles and
e) introducing such phospholipid vesicles into the bloodstream of the body to obtain the movement of such phospholipid vesicles to the tumor in the body.

10. A method of targeting a tumor in a body with an agent for the diagnosis or treatment thereof comprising the steps of:

a) providing vesicles comprising chemically pure phospholipid molecules having positively charged amino groups incorporated therewith;
b) introducing such positively charged vesicles to the bloodstream of the body to block macrophages in the body;
c) providing small vesicles of less than 2000 Å comprising chemically pure phospholipid molecules having incorporated therein the agent for diagnosis Dr treatment; and
d) introducing the small vesicles with agent into the bloodstream of the body subsequent to the blocking of macrophages to obtain movement of the small vesicles to the tumor.

11. A method as set forth in claim 10 wherein charged molecules are also incorporated into said small vesicles and wherein the charged molecules may be positively charged and may constitute stearylamine, or aminomannose or aminomannitol derivatives of cholesterol, or may be negatively charged and may constitute dicetyl phosphate and wherein the charged molecules extend externally from the phospholipid vesicles.

12. A method as set forth in claim 11 wherein the phospholipid constitutes distearoyl phosphatidyl choline.

13. A method as set forth in claim 10 wherein the charged molecules are selected from a group consisting of stearylamine, aminomannose or aminomannitol derivatives of cholesterol and dicetyl phosphate.

14. A method according to claim 10 wherein the agent is a label which can be detected in vivo and further comprising the step of determining the location of the small vesicles in the body by detecting said label.

15. A method of targeting tumors in a body, comprising the steps of:

a) providing small vesicles of less than 2000 Å comprising neutral phospholipids;
b) adding cholesterol to such neutral phospholipid vesicles;
c) incorporating charged molecules into such neutral phospholipid vesicles in the range of approximately 0% to 20% by weight of the charged molecules and the remainder constituting the phospholipids.
d) incorporating a drug into such phospholipid vesicles; and
e) introducing such phospholipid vesicles into the body to obtain the targeting of such phospholipid vesicles to the specific locations of the tumor in the body.

16. A method of targeting tumors in a body, including the steps of:

a) providing small vesicles of less than 2000 Å comprising chemically pure phospholipid molecules;
b) modifying a portion of the phospholipid vesicles to provide for the blockage of the macrophages in the body by such modified phospholipid vesicles;
c) initially introducing the modified phospholipid vesicles to the bloodstream of the body to block uptake by the macrophages in the body;
d) incorporating an agent for diagnosis or treatment of the tumor in a second portion of the vesicles; and
e) subsequently introducing the second portion of phospholipid vesicles to the bloodstream of the body to obtain a movement of lipid vesicles and agent to the tumor in the body.

17. A method of targeting tumors in the body, including the steps of:

a) providing small, chemically pure phospholipid vesicles of less than 2000 Å;
b) incorporating monosaccharide derivatives of cholesterol into the phospholipid vesicles so that the monosaccharides extend externally from the vesicles;
c) incorporating into the vesicles an agent for the diagnosis or treatment of the tumor; and
d) introducing the monosaccharide-containing lipid vesicles into the bloodstream to obtain the movement of said vesicles to the location of the tumor in the body.

18. A method as set forth in claim 17 wherein the phospholipid constitutes distearoyl phosphatidyl choline.

19. A method as set forth in claim 18, including the step of blocking the macrophages in the body before introducing said phospholipid vesicles into the body.

20. A method as set forth in claim 9 wherein the phospholipids in the phospholipid vesicles constitute distearoyl phosphatidylcholine and wherein the charges in the charged phospholipid vesicles are provided by aminomannose or aminomannitol derivatives of the cholesterol and are coupled to the phospholipid vesicles.

21. A method as set forth in claim 20 wherein the charged molecules extend externally of the phospholipid vesicles.

22. A method as set forth in claim 21 wherein the charged molecules are selected from the group consist-

ing of stearylamine, dicetylphosphate, and aminomannose and aminomannitol derivatives of cholesterol.

23. A method as set forth in claim **16** wherein the modified phospholipid vesicles are charged.

24. A method as set forth in claim **16** wherein cholesterol is coupled to the phospholipid vesicles.

25. A method as set forth in claim **24** wherein aminomannose or aminomannitol derivatives of cholesterol are coupled to the phospholipid vesicles to modify the phospholipid vesicles and wherein the phospholipid vesicles are charged by a material selected from the group consisting of stearylamine, dicetyl phosphate and aminomannose and aminomannitol derivatives of cholesterol.

26. A method as set forth in claim **25** wherein charged molecules in the range of 0% to 20% are coupled to the phospholipid vesicles to modify the phospholipid vesicles for blocking the uptake of other phospholipid vesicles by the macrophages in the body and the charged molecules extend externally from the phospholipid vesicles.

27. A method as set forth in claim **26** wherein the phospholipids constitute distearoyl phosphatidylcholine.

28. A method as set forth in claim **16** wherein the phospholipid vesicles targeted to the specific locations in the body are labeled to identify such specific locations.

29. A method as set forth in claim **17**, including the step of: labelling the monosaccharide containing lipid vesicles to identify the specific locations of the tumor in the body.

30. A method as set forth in claim **29** wherein the monosaccharide derivative of cholesterol has a range of approximately 0% to 20% by weight of the monosaccharides and the remaining weight constituting the phospholipids.

31. A method as set forth in claim **1**, wherein radioactive labels are provided in the phospholipid vesicles for subsequent identification of the specific locations of the tumor; and a chemical is chelated to such labels with a weak bond to provide for displacement by protein components in the body when the phospholipid vesicles have targeted the specific locations of the tumor.

32. A method as set forth in claim **6** wherein a drug or radionuclide is disposed within the micellular particles to be released at the specific locations of the tumor for treating the body at such locations.

33. A method as set forth in claim **31** including the step of: chelating nitrilotriacetic acid to Indium-111 to provide a weak bond between the nitrilotriacetic acid and the Indium-111 and to provide for a displacement of the nitrilotriacetic acid by proteins in the body at the specific location of the tumor.

34. A method as set forth in claim **5** wherein the phospholipid particles are labeled to facilitate the identification of the specific locations of the tumor to which the phospholipid particles are targeted.

35. The method of claims **6** or **32** in which distearoyl phosphatidylcholine and cholesterol are included in said particles in a 2:1 molar ratio.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,435,989

DATED : July 25, 1995

INVENTOR(S) : Cary A. Presant, et al

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 12, line 8, after "comprising" add --chemically pure--.
Column 12, line 9, after "such" add --chemically pure--.
Column 12, line 11, after "such" add --chemically pure--.
Column 12, line 15, after "such" add --chemically pure--.
Column 12, line 17, after "such" add --chemically pure--.
Column 12, line 18, after "such" add --chemically pure--.

Signed and Sealed this

Fourteenth Day of October, 1997

Attest:

Bruce Lehman

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,435,989

DATED : July 25, 1995

INVENTOR(S) : Cary A. Presant, et al

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In the drawings, Sheet 1 of 3, Figure 1, delete in its entirety and replace with new Figure 1.

ORGAN	% INJECTED DOSE PER GRAM OF TISSUE (± S.D)			
	FREE ^{111}In-NTA (n=2)	^{111}In-NTA DS PC:Ch (n=4)	^{111}In-NTA DS PC:Ch:SA (n=4)	^{111}In-NTA DS PC:Ch:DP (n=4)
TUMOR	2.4	18.5 ± 4.7	6.2 ± 2.1	11.9 ± 2.0
BLOOD	0.30	6.6 ± 1.6	0.95 ± 0.34	13 ± 0.4
LIVER	3.1	14.6 ± 1.7	28.5 ± 2.2	16.6 ± 1.6
SPLEEN	2.5	18.8 ± 3.3	43.8 ± 5.2	39.3 ± 3.4
KIDNEY	10.8	6.8 ± 0.6	6.8 ± 0.6	12.7 ± 3.5
LUNG	1.8	6.0 ± 1.5	1.8 ± 0.1	3.0 ± 0.5
BONE	2.4	3.9 ± 1.5	2.6 ± 0.6	4.8 ± 0.5

FIG. 1

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,435,989
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INVENTOR(S) : Cary A. Presant, et al

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In the drawings, Sheet 2 of 3, Figure 2, delete in its entirety and replace with new Figure 2.

ORGAN	% INJECTED DOSE PER GRAM OF TISSUE (± S.D) DS PC:Ch:AM (4:1:1) AM (n=2)	DS PC:Ch:AM (8:3:1) AM/2 (n=3)	DS PC:Ch:AML (4:1:1) AML (n=3)
TUMOR	0.91	1.0 ± 0.5	1.7 ± 0.2
BLOOD	0.23	0.24 ± 0.07	0.30 ± 0.02
LIVER	29.6	40.5 ± 6.9	17.4 ± 1.4
SPLEEN	49.0	74.4 ± 28.6	56.0 ± 10.9
KIDNEY	4.62	2.5 ± 0.5	6.5 ± 1.0
LUNG	3.2	1.5 ± 0.9	4.7 ± 2.0
BONE	1.7	1.2 ± 0.6	3.0 ± 0.5

FIG.2